**PATENT**

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**
**BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES**

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|---|---|---|
| *In re* Application of | ) | |
| | ) | Group Art Unit: 1643 |
| Schneck *et al.* | ) | |
| | ) | Examiner: C. H. Yaen |
| Serial No. 09/642,660 | ) | |
| | ) | |
| Filed: August 22, 2000 | ) | Atty. Docket No. 01107.00042 |

FOR: **CELL COMPOSITIONS COMPRISING MOLECULAR COMPLEXES THAT MODIFY IMMUNE RESPONSES**

## BRIEF ON APPEAL

U.S. Patent and Trademark Office
Randolph Building
401 Dulany Street
Alexandria, VA 22314

Sir:

A Notice of Appeal was filed on September 5, 2006. Please charge the fee for filing this Brief and any other fee which may be due to our Deposit Account No. 19-0733.

## REAL PARTY IN INTEREST

The real party in interest in this application is The Johns Hopkins University.

## RELATED APPEALS AND INTERFERENCES

There is one related appeal. A Brief on Appeal was filed June 29, 2006 in related application Serial No. 09/954,166. Both Serial No. 09/954,166 and the present application claim priority to Serial No. 08/828,712 filed March 28, 1997 and to Serial No. 60/014,367 filed April 28, 1996.

There are no related interferences.

## STATUS OF CLAIMS

Claims 1-27 and 33-50 are canceled. Claims 28-32 and 51-60 are pending. Claims 59 and 60 are allowed. Claims 28-32 and 51-58 are rejected. Appellants appeal the rejection of claims 28-32 and 51-58.

## STATUS OF AMENDMENTS AFTER FINAL REJECTION

Allowed claims 59 and 60 were amended to be in independent form in an amendment filed on July 20, 2006 under 37 C.F.R. § 1.116. The Advisory Action mailed August 24, 2006 indicates the amendment was entered.

## SUMMARY OF CLAIMED SUBJECT MATTER

Independent claim 28 is directed to a composition comprising a cell in which a molecular complex is bound to the surface of the cell. Page 4, lines 25-26. The molecular complex comprises at least two first fusion proteins and at least two second fusion proteins. Page 4, lines

26-27. Each of the two first fusion proteins comprises an immunoglobulin heavy chain, wherein the immunoglobulin heavy chain comprises a variable region, and an extracellular portion of a first transmembrane polypeptide. Page 4, lines 27-29. Each of the two second fusion proteins comprises an immunoglobulin light chain and an extracellular portion of a second transmembrane polypeptide. Page 4, lines 29-31. The at least two first fusion proteins and the at least two second fusion proteins associate to form the molecular complex. Page 4, line 31 to page 5, line 1. The molecular complex comprises two ligand binding sites. Page 5, lines 1-2. Each ligand binding site is formed by the extracellular domain of a first transmembrane polypeptide and the extracellular domain of a second transmembrane polypeptide. Page 5, lines 2-3. The affinity of the molecular complex for a cognate ligand is increased at least two-fold over a dimeric molecular complex consisting of the first and the second fusion protein. Page 5, lines 3-5.

## GROUNDS OF REJECTION TO BE REVIEWED

1. Whether claims 32 and 56-58 are sufficiently described under 35 U.S.C. § 112 ¶ 1.
2. Whether claims 28-32 and 51-55 are patentable under 35 U.S.C. § 103(a).

## ARGUMENT

1. The specification fully describes claims 32 and 56-58.

a. Legal Standards

The first paragraph of 35 U.S.C. § 112 requires the specification to provide a written description of the claimed invention:

> [t]he specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same, and shall set forth the best mode contemplated by the inventor of carrying out his invention.

The purpose of the written description requirement is to ensure that the specification conveys to those skilled in the art that the applicants possessed the claimed subject matter as of the filing date sought. *Vas-Cath Inc. v. Mahurkar*, 935 F.2d 1555, 1563-64, 19 U.S.P.Q.2d 1111, 1117 (Fed. Cir. 1991).

Whether the specification meets the written description requirement for a claimed invention is a question of fact. *Vas-Cath,* 935 F.2d at 1563, 19 U.S.P.Q.2d at 1116. The specification is directed to those skilled in the art. *Vas-Cath*, 935 F.2d. at 1563-1564, 19 U.S.P.Q. at 1115. Thus, the knowledge of those skilled in the art must be considered when determining whether a specification meets the written description requirement. *In re Wright*, 866 F.2d 422, 425, 9 U.S.P.Q.2d 1649, 1651 (Fed. Cir. 1989). The teachings of the specification must be considered as a whole. *Id.*, 9 U.S.P.Q.2d at 1651.

A specification adequately describes a genus to the skilled artisan if it permits the artisan to "visualize or recognize members of the genus." *University of California v. Eli Lilly and Co.*, 119 F.3d 1559, 1568, 43 U.S.P.Q.2d 1398, 1406 (Fed. Cir. 1997). The existing knowledge in a

particular field, the extent and content of the prior art, and the maturity of the science at issue must be considered when determining what is needed to support generic claims to biological subject matter. *Capon v. Eshhar*, 418 F.3d 1349, 1359, 76 U.S.P.Q. 2d 1078, 1085 (Fed. Cir. 2005).

It is black letter law that "[t]he description need only describe in detail that which is new or not conventional in the art." M.P.E.P. § 2163(II)(A)(3)(a), citing *Hybritech v. Monoclonal Antibodies*, 802 F.2d 1367, 1384, 231 U.S.P.Q. 81, 94 (Fed. Cir. 1986). *See also* the U.S. Patent and Trademark Office's own Revised Interim Written Description Guidelines Training Materials at page 4: "It is assumed at this point in the analysis that the specification has been reviewed and an appropriate search of the claimed subject matter has been conducted. It is also assumed that the examiner has identified which features of the claimed invention are conventional taking into account the body of existing prior art."

b. <u>The specification satisfies the legal requirements for a written description of claims 32 and 56-58 for at least two reasons</u>.

The recited molecular complex contains two ligand binding sites. Dependent claims 32 and 56-58 recite that an identical antigenic peptide is bound to each of the ligand binding sites recited in independent claim 28. The Examiner contends that the written description requirement for the genus "antigenic peptides" is not met because "the specification has not provided a representative number of species in a highly divergent genus so that it can be used to encompass the broad scope of the peptides claimed." Final Office Action mailed July 14, 2004 at page 3. The Examiner's position is that only a disclosure of a "representative number" of specific antigenic peptides would satisfy the written description requirement for the recited genus. This position is legally incorrect for at least two reasons.

First, in contrast to the molecular complex itself, "antigenic peptides" are neither new nor unconventional in the art; therefore, they do not require explicit description to be understood by those skilled in the art. It has long been known in the art that antigenic peptides are formed by the processing of internalized proteins in endosomal/lysosomal vesicles to peptides which can be presented by antigen presenting cells. See Abbas *et al.*, Cellular and Molecular Immunology, 3rd ed., W.B. Saunders Company, Philadelphia, 1997, pages 125-37 (Attachment 1). A PubMed search for "antigenic peptide" identified 848 references (Attachment 2), dating back as far as 1979 (*e.g.*, Smith; Attachment 3). A PubMed search for "peptide antigen" identified 517 references (Attachment 2), dating back as far as 1965 (*e.g.*, Akuzawa & Tsuchiya; Attachment 4). In fact, as the Examiner acknowledges, "just about any peptide sequence to some extent is considered 'antigenic.'" Final Office Action at page 3, lines 18-19. Thus, taking into account the body of existing prior art, the antigenic peptides recited in claims 32 and 56-58 are features of the claimed invention which are conventional and therefore need not be described. *Hybritech*, 802 F.2d at 1384, 231 U.S.P.Q. at 94; M.P.E.P. § 2163(II)(A)(3)(a).

Second, an explicit description of individual species is not the only way to provide written description of a recited genus:

> The written description requirement for a claimed genus may be satisfied through sufficient description of a representative number of species by actual reduction to practice, reduction to drawings, or by disclosure of relevant, identifying characteristics, *i.e.*, structure or other physical and/or chemical properties, by functional characteristics coupled with a known or disclosed correlation between function and structure, or by a combination of such identifying characteristics . . . .

U.S. Patent and Trademark Office's Written Description Guidelines, 66 Fed. Reg. 1099, 1106 (January 5, 2001) (internal references omitted), cited with approval in *Enzo Biochem, Inc. v.*

*Gen-Probe Incorporated*, 296 F.3d 1316, 1325, 63 U.S.P.Q.2d 1609, 1613 (Fed. Cir. 2002). There is a known correlation between the structure and function of antigenic peptides. An antigenic peptide, for example, has the function of binding to the peptide binding site formed by two extracellular domains of a class II MHC molecule. It also has the function of binding to the peptide binding site formed by two extracellular domains of a T cell receptor. Structural characteristics of antigenic peptides which correlate with these two functions are known. See Abbas *et al.*, Cellular and Molecular Immunology, 3rd ed., W.B. Saunders Company, Philadelphia, 1997, pages 105-07 ("These features of the peptide-MHC interaction can now be explained in precise structural terms"; p. 106) and page 147, Table 7-2 (identifying MHC-binding and TCR-binding residues in peptide antigens) (Attachment 5). Thus, the genus of "antigenic peptides" is adequately described under the U.S. Patent and Trademark Office's own guidelines.

c. None of the case law the Examiner cites applies to the written description of the recited antigenic peptides.

The Examiner cites several cases, but none applies to the written description of the recited antigenic peptides. First, the Examiner draws an analogy between the recited genus of antigenic peptides and the genus of nucleic acids claimed in *Lilly*:

> In deciding *The Regents of the University of California v. Eli Lilly* 43 USPQ2d 1398 (CAFC 1997), the Federal Circuit held that a generic statement that defines a genus of nucleic acids *by only their functional activity* does not provide an adequate written description of the genus. By analogy, a generic statement that defines a genus of "antigenic peptides" by only their common ability [sic] bind to the peptide binding site of an MHC or to the peptide binding site of a T-cell receptor TCR, as argued in the response filed 3/8/2005 does not serve to adequately describe the genus as a whole.

Final Office Action at page 3, lines 1-7. The analogy is faulty. *Lilly* addressed what is required for a written description or conception of new genetic material. Applicants do not claim a new genetic material. Applicants claim a composition comprising a cell to which a molecular complex is bound. The components of the recited molecular complex – including the recited antigenic peptides – are known in the art.

Second, the Examiner cites *University of Rochester v. G.D. Searle Co.*, for the proposition that "generalized language may not suffice if it does not convey the detailed identity of an invention." Final Office Action at page 3, lines 19-21, quoting *University of Rochester v. G.D. Searle Co.*, 358 F.3d 916, 923, 69 U.S.P.Q.2d 1886, 1892 (Fed. Cir. 2004), *reh'g en banc denied*, 375 F3d. 1303, 71 U.S.P.Q.2d 1545 (Fed. Cir. 2004), *cert. denied* 543 U.S. 1015 (2004). Again, "antigenic peptides" are not the invention. They are a class of molecules well known in the art which can be employed in the claimed invention.

Third, the Examiner cites *Noelle v. Lederman*, 355 F.3d 1343, 69 U.S.P.Q.2d 1508 (Fed. Cir. 2004) for the proposition that "a patentee of a biotechnological invention cannot necessarily claim a genus after only describing a limited number of species because there may be unpredictability in the results obtained from species other than those specifically enumerated." Final Office Action mailed April 20, 2006 at page 4, lines 1-6. Again, "antigenic peptides" are not the claimed invention. Nor, as the Examiner himself acknowledges, is it unpredictable to obtain an antigenic peptide: "just about any peptide sequence to some extent is considered 'antigenic.'" Final Office Action mailed July 14, 2004 at page 3, lines 18-19.

None of the cited cases are apt. "Antigenic peptides" are not the claimed invention. Dependent claims 32 and 56-58 merely recite a well-known class of molecules – antigenic peptides – which are bound to ligand binding sites of the recited molecular complex.

The rejection under 35 U.S.C. § 112 ¶ 1 has no legal foundation. The specification adequately describes the subject matter of dependent claims 32 and 56-58 because it describes the new and unconventional subject matter encompassed within those claims. There is no legal requirement to describe that which is conventional. *Hybritech*, 802 F.2d at 1384; 231 U.S.P.Q. at 94; M.P.E.P. § 2163(II)(A)(3)(a). The Board should reverse the rejection.

2. <u>Claims 28-32 and 51-55 are not *prima facie* obvious.</u>

a. <u>Legal Standards</u>

Section 103(a) of 35 U.S.C. states:

> A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains.

Obviousness under 35 U.S.C. § 103(a) is a question of law based on several factual inquiries: "Under § 103, the scope and content of the prior art are to be determined; differences between the prior art and the claims at issue are to be ascertained; and the level of ordinary skill in the pertinent art resolved." *Graham v. John Deere Co.*, 383 U.S. 1, 17 (1966).

The Examiner bears the burden of making factual findings to establish a *prima facie* case of obviousness. M.P.E.P. § 2142. The *prima facie* case requires three elements. First, the cited prior art must teach or suggest all the claim limitations. *In re Royka*, 490 F.2d 981, 985, 180 U.S.P.Q. 580, 583 (C.C.P.A. 1974). Second, the facts must establish one of ordinary skill would have been motivated to combine the cited references. *In re Linter*, 458 F.2d 1013, 1016, 173 U.S.P.Q. 560, 562 (C.C.P.A. 1972). Third, the facts must establish that one of ordinary skill in

the art would have had a reasonable expectation that the asserted combination or modification would be successful. *In re Merck & Co.*, 800 F.2d 1091, 1097, 231 U.S.P.Q. 375, 379-80 (Fed. Cir. 1986).

The cited references must be considered in their entireties, including portions of the references which would have led the ordinary artisan away from the claimed invention. *W.L. Gore & Associates, Inc. v. Garlock, Inc.*, 721 F.2d 1540, 1550, 220 U.S.P.Q. 303, 310 (Fed. Cir. 1983), *cert. denied*, 469 U.S. 851 (1984). It is black letter law that hindsight use of an applicant's specification is improper. *In re Kotzab*, 217 F.3d 1365, 1371, 55 U.S.P.Q.2d 1313, 1317 (Fed. Cir. 2000).

b. The Rejection

The Examiner cites four references: Matsui[1] (the primary reference), Dal Porto,[2] Chang,[3] and Harris.[4] The Examiner cites Matsui as teaching that the interaction between monovalent TCRs and MHC heterodimers has been difficult to study directly because it is a low affinity interaction. Office Action mailed August 11, 2005 at page 5 ¶ a. The Examiner cites Dal Porto as disclosing high affinity divalent class I MHC/IgG molecules which have nanomolar affinity for T cell receptors and which, in contrast to monovalent MHC class I molecules, inhibit lysis of target cells. *Id.* at pages 5-6 ¶ b. The Examiner cites Chang as teaching that "the fusion of peptide sequences known to form unique, heterodimeric coiled-coils to the C-termini of the TCR α and β extracellular segments promotes heterodimer formation over homodimer formation." *Id.*

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[1] Matsui *et al.*, *Proc. Natl. Acad. Sci. U.S.A. 91*, 12862-66, December 1994.

[2] Dal Porto *et al.*, *Proc. Natl. Acad. Sci. U.S.A. 90*, 6671-75, 1993.

[3] Chang *et al.*, *Proc. Natl. Acad. Sci. USA 91*, 11408-412, 1994.

at page 6 ¶ c. The Examiner cites Harris as demonstrating that binding domains, including cell surface receptors, can be fused via a linker to the N-terminus of heavy and light chain variable regions "and the fusion proteins retain binding activity." *Id.* at page 6 ¶ d.

The thrust of the rejection is that the recited molecular complex would have been obvious because high affinity, divalent soluble TCR and class II molecules are desirable (Matsui), MHC class I/Ig molecules have higher affinity for TCRs than do monovalent MHC class I molecules (Dal Porto), heterodimer formation can be facilitated using leucine zippers (Chang), and binding domains which are fused to the N-termini of heavy and light chains retain their binding function (Harris).

c. The Examiner did not evaluate the cited references under the proper legal standards.

The Examiner did not evaluate the cited references under the proper legal standards. The rejection set forth above ignores large portions of each reference, including teachings in Matsui, Dal Porto, and Chang that explicitly teach away from the invention. Instead, using the specification as a template, the Examiner selected isolated teachings of the cited references, modified them, and combined them without regard to what each of the references teaches as a whole. This is clear legal error. *Gore*, 721 F.2d at 1550, 220 U.S.P.Q. at 310; *Kotzab*, 217 F.3d at 1371, 55 U.S.P.Q.2d at 1317.

The remainder of this Brief analyzes the cited references under the proper legal standards for determining obviousness. The analysis demonstrates that the cited references – even if, *arguendo*, properly combined – do not render claims 28-31 and 51-55 *prima facie* obvious.

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[4] Harris *et al.*, WO 94/09131, April 28, 1994.

*Matsui.* The primary reference, Matsui, addresses the problem of how to obtain direct measurements of the binding kinetics between a soluble TCR and a peptide presented by a soluble MHC molecule. Matsui acknowledges that soluble TCRs are available and that several studies have determined that the binding affinities between soluble TCRs and peptides presented by soluble MHC molecules are relatively low ($K_d$ of 4-6 x $10^{-5}$ M, $10^{-4}$-$10^{-7}$ M, and $10^{-5}$ M, respectively). Matsui points out that these measurements were indirect <u>and depend on live cells</u>, which is a disadvantage: "However, none of these studies give direct information about the kinetics of the molecular interactions and are dependent on live cells, thus greatly limiting the range of conditions (temperature, ionic strength, etc.) that can be assessed." Matsui at page 12862. Matsui teaches use of surface plasmon resonance to overcome the disadvantages of indirect measurements so that low affinity interactions between a soluble TCR and a peptide presented by a soluble MHC molecule can be studied directly. Matsui's method is designed to avoid the use of a cell.[5]

*Dal Porto.* Dal Porto teaches a class I MHC/IgG complex which comprises an immunoglobulin molecule and two MHC class I molecules (Figure 1B):[6]

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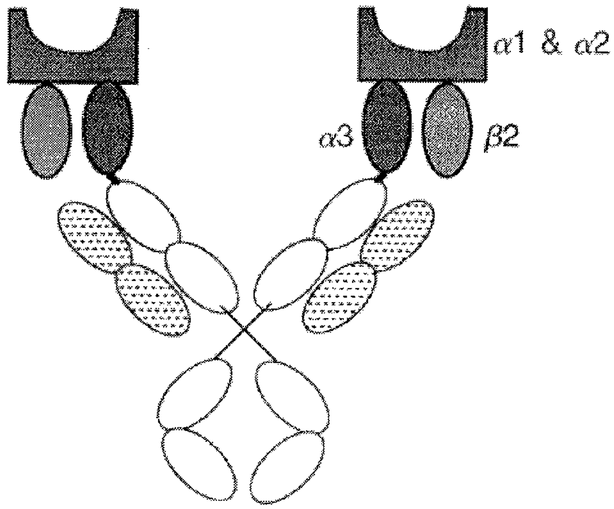
[5] In contrast, all the appealed claims are directed to compositions which comprise a cell.

[6] As indicated in Figure 1B, a class I MHC molecule comprises an $\alpha$ chain of three segments ($\alpha 1$, $\alpha 2$, and $\alpha 3$) and a $\beta_2$ microglobulin subunit.




The Dal Porto complex comprises two of a single species of fusion protein: an immunoglobulin heavy chain (white) fused to the $\alpha_3$ subunit of the α chain of a class I MHC molecule (dark grey).[7] Neither the immunoglobulin light chains (stippled) nor the $\beta_2$ microglobulin subunits (light grey) are part of a fusion protein. The $\beta_2$ microglobulin subunit associates with the MHC class I α chain as it normally does in a native class I MHC molecule. The immunoglobulin light chain associates with the immunoglobulin heavy chain as it does in a native immunoglobulin molecule.

Dal Porto teaches that the "divalent MHC/IG molecules are good candidates for soluble high-affinity MHC-like molecules that could be used to selectively suppress specific T-cell responses." Page 6675, sentence bridging columns 1 and 2. Dal Porto neither teaches nor suggests binding the disclosed soluble molecules to the surface of a cell.

*Chang*. Chang teaches a method of making a soluble TCR. Chang fused segments of 30 amino acids to the carboxyl termini of TCR α and β extracellular domains via a flexible linker. The fused segments associate to form a leucine zipper, which facilitates pairing of the TCR α and

[7] In contrast, all the appealed claims require <u>two</u> species of fusion proteins.

β subunits. Page 11408, col. 2. Chang teaches that use of leucine zipper components "should be broadly useful in the efficient production and purification of TCRs as well as other heterodimeric proteins." Abstract. See also page 11412, paragraph bridging columns 1 and 2:

> In principle, it should now be possible to facilitate association of any type of naturally occurring heterodimeric structure including, for example, MHC class II α and β subunits or CD8α and CD8β components. . . . In addition, it should also be possible to force association between proteins that may never or only transiently come in contact with one another, thereby offering a means to better understand regulatory events affecting cellular activation, cell cycle control, gene transcription, or cellular differentiation.

Paragraph bridging columns 1 and 2 of page 11412. There is no teaching or suggestion in Chang to use anything other than leucine zipper components to associate heterodimeric proteins. There is nothing in Chang which teaches or suggests binding any type of heterodimeric proteins to the surface of a cell.[8]

*Harris*. Harris teaches "recombinant bispecific (heterodimeric) and/or monodimeric bivalent specific binding proteins, for example antibodies, in which the specific association of the component modules is accomplished by using the recognition and natural homo- or hetero-dimerization of additionally fused associating domains." Page 8, lines 13-19. The bulk of the Harris disclosure relates to antibodies. The disclosed purpose of the binding proteins is to provide high affinity antibodies, particularly bispecific antibodies, which are not immunogenic in humans and which do not have the undesirable effector functions of complete antibody molecules: "the effector functions intrinsic to complete antibody molecules (such [as] Fc

[8] In contrast, the dimerizations which occur in the recited molecular complexes are those which naturally occur between immunoglobulin heavy chains and between immunoglobulin heavy and light chains.

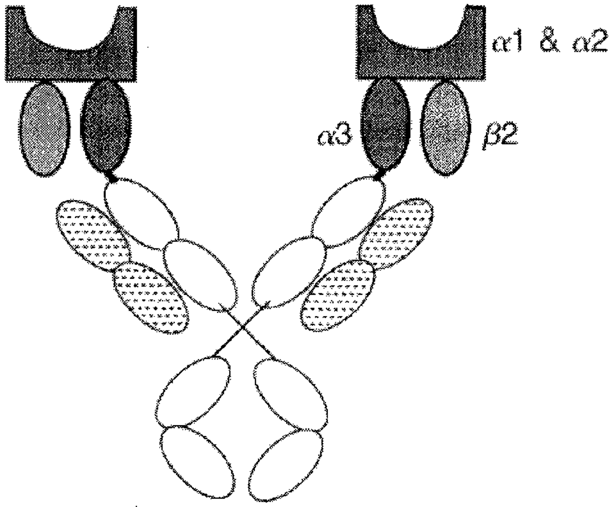
receptor and complement binding) have led to undesirable interactions." Harris, paragraph bridging pages 1 and 2. [9]

d. The combination of cited references does not teach or suggest all elements of the claimed subject matter.

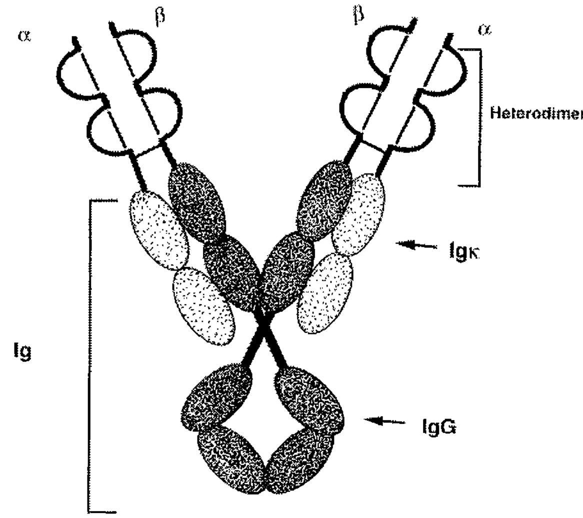
The claimed subject matter is a composition comprising a cell. A molecular complex with particular recited features is bound to the surface of the cell. Even if, *arguendo*, the combination of cited references taught or suggested the recited molecular complex – which it does not – the combination does not teach or suggest binding the molecular complex to the surface of a cell. The Examiner has not addressed this aspect of the claimed invention at all. This omission alone is sufficient to defeat the alleged *prima facie* case of obviousness. See M.P.E.P. § 2142 ("the prior art reference (or references when combined) must teach or suggest all the claim limitations").

Moreover, contrary to the Examiner's characterization of Dal Porto's molecule and the recited molecular complex, the two differ significantly. The molecule of Dal Porto (left; Fig. 1B) and an embodiment of the recited molecular complex (right; specification Fig. 1B) are illustrated below:

[9] In contrast, all the appealed claims require the portion of the immunoglobulin heavy chain which has the effector function.




Dal Porto, Fig. 1B




specification, Fig. 1B

As described above, Dal Porto's complexes comprise a single species of fusion protein which consists of the immunoglobulin heavy chain (white) and the MHC class I α chain (consisting of α1, α2, and α3 segments; dark grey). The $\beta_2$ microglobulin subunit (light grey) associates with the α chain as it normally does in a native class I MHC molecule. The immunoglobulin light chain (stippled) associates with the immunoglobulin heavy chain as it does in a native immunoglobulin molecule. Neither the immunoglobulin light chains nor the $\beta_2$ microglobulin subunits are part of a fusion protein. Thus, Dal Porto's complex contains only a single species of fusion protein.

In contrast, the recited molecular complexes comprise two types of fusion proteins. One fusion protein of the recited complex comprises an extracellular portion of a first transmembrane polypeptide ("β" in Fig. 1B) fused to an immunoglobulin heavy chain. The other species of fusion protein comprises an extracellular portion of a second transmembrane polypeptide ("α" in

Fig. 1B) fused to an immunoglobulin light chain. In Dal Porto's complex, neither the $\beta 2$ microglobulin nor either of the light chains is part of a fusion protein.

Thus, contrary to the Examiner's assertion in the Final Office Action, the recited molecular complexes do not "merely differ from the molecular complex of Dal Porto *et al.* by substitution of the extracellular domains (alpha and beta subunits) of the TCR and class II MHC molecules in place of the class I MHC portion of the molecule of Dal Porto *et al.*" Final Office Action at page 8 ¶ 1. Such a substitution – which Dal Porto neither teaches nor suggests – would not have formed the recited molecular complex, which requires two distinct species of fusion proteins. In fact, modifying Dal Porto's molecule to arrive at a molecular complex such as that in Figure 1B of the specification, for example, requires <u>two</u> significant modifications: (1) fusing the extracellular domain of a first transmembrane polypeptide to the immunoglobulin heavy chain in place of the class I MHC $\alpha$ chain and (2) fusing the extracellular domain of a second transmembrane polypeptide to the immunoglobulin's light chain. None of the cited references teaches or suggests these modifications.

e. <u>One of ordinary skill in the art would have had no motivation to select isolated elements of the cited references, modify them, and combine them as the Examiner asserts</u>.

The relevant question is not whether Matsui – or any other teaching in the art – would have motivated the ordinary artisan to make a divalent soluble MHC or TCR molecule as the Examiner contends. The relevant question is whether Matsui, in view of Chang, Harris, and Dal Porto, would have motivated one of ordinary skill <u>to make the molecular complex recited in claims 28-31 and 51-55 **and to bind it to the surface of a cell**</u>. The answer is no.

Matsui simply teaches a method to obtain direct measurements of interactions between a soluble TCR and a peptide presented by a soluble MHC complex. Matsui solves the problem of measuring interactions between these low-affinity binding partners; the solution involves a cell-free system. Thus, Matsui as a whole would not have motivated one of ordinary skill to make soluble TCR or MHC molecules with higher binding affinities and to bind the soluble molecules to the surface of a cell. Matsui in fact plainly teaches away from using cells.

Chang teaches no other method of associating polypeptides other than by using leucine zipper components. Polypeptides associated via a leucine zipper as taught in Chang are stabilized by interdigitation of leucine residues on two protein alpha-helices. The fusion proteins of the recited molecular complex, however, comprise immunoglobulin chains. Immunoglobulin chains are not held together with leucine zippers. Chang's teaching of leucine zippers therefore would not have motivated an ordinary artisan to use immunoglobulin chains, which have a very different secondary structure. As is known in the art, all domains of immunoglobulin chains such as those recited in the claims contain two layers of $\beta$-pleated sheet which have three or four strands of antiparallel polypeptide chain which interact and that immunoglobulin chains are further held together with disulfide bonds.[10] Moreover, Chang explicitly teaches soluble molecules; it contains no teaching or suggestion to bind any molecules to the surface of a cell, thereby rendering them insoluble.

The recited molecular complex comprises an immunoglobulin heavy chain; an immunoglobulin heavy chain comprises both variable and constant regions. Harris explicitly teaches one of ordinary skill not to include constant regions of an immunoglobulin molecule in its binding proteins. In fact, use of an immunoglobulin heavy chain would render the Harris

binding proteins unsatisfactory for one of their intended purposes (to avoid undesirable effector functions). There is, therefore, no suggestion in Harris to include both heavy and light immunoglobulin chains, which are present in molecular complexes of the invention. *In re Gordon*, 733 F.2d 900, 902, 221 U.S.P.Q. 1125, 1127 (Fed. Cir. 1984); M.P.E.P. § 2143.01(V) ("If the proposed modification would render the prior art invention being modified unsatisfactory for its intended purpose, then there is no suggestion or motivation to make the proposed modification.").

Furthermore, those of skill in the art at the April 28, 1996 priority date of this application knew that no particular manipulation was needed to cause the extracellular domains of MHC class II molecules or TCRs to associate to form functional peptide binding sites. It was well known that the two extracellular domains of TCR molecules or of class II MHC molecules will associate to form a peptide binding site in the absence of their transmembrane domains. That is, the ordinary artisan knew that one extracellular domain need not be anchored in any particular orientation relative to the other extracellular domain in order for the two extracellular domains to associate and form a functional peptide binding site.

For example, U.S. Patent 5,723,309 (Attachment 8) discloses soluble TCR molecules which contain the extracellular domains but not the transmembrane domains of each polypeptide chain: "VγCγ/VδCδ soluble T receptors are also produced by co-transfecting, into a host cell, DNA sequences encoding the γ and δ submits [sic; subunits] of the Tγδ receptor from which the transmembrane portion of the Tγδ receptor has been deleted." Col. 2, lines 48-51. When the transfected DNA is expressed, a soluble TCR containing both extracellular domains is secreted

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[10] Abbas *et al.*, eds., Cellular and Molecular Immunology, 3d ed., pages 41-43 (Tab 6).

into the supernatant: "[T]he soluble $\gamma\delta$ hetero-dimers were clearly detected by IRMA (radioimmunological assay) in the supernatants of CHO cells co-transfected with soluble $\gamma$ and soluble $\delta$ assembly products ($\gamma\delta$sFS-CHO) when pairs of antibodies specific for V$\delta$2/C$\gamma$ or V$\delta$2/V$\gamma$9 were used" Col. 8, lines 43-50. The soluble TCR molecules contain a functional peptide binding site and can be used diagnostically (col. 5, lines 3-13) and therapeutically (col. lines 57-60).

U.S. Patent 5,583,031 (Attachment 9) discloses a soluble class II MHC molecule that contains extracellular domains of each polypeptide chain but not the transmembrane domains and that can bind an antigenic peptide:

> Class II histocompatibility proteins are expressed as $\alpha\beta$ heterodimers by insect cells (*Spodoptera frugiperda*, fall armyworm) infected with recombinant baculoviruses. The viruses carry genes coding for the $\alpha$ and for the $\beta$ subunits of the histocompatibility protein. The protein can be produced in a membrane-associated form, or in a secreted, soluble form by alteration of the carboxy-terminus. Like the mammalian cells from which histocompatibility proteins are conventionally isolated, the insect cells glycosylate and correctly assemble the histocompatibility protein, but, unlike the mammalian cells, they do not load the binding site with tightly bound endogenous peptides. The proteins are isolated from insect cells as empty molecules by immunoaffinity and ion-exchange procedures. Antigenic peptide is loaded onto the purified molecule in vitro, and the 1:1 complex of peptide and histocompatibility protein is isolated.

Col. 5, lines 9-24.

As evidenced by these two patents, the ordinary artisan would not have thought that any particular manipulation was necessary to permit the extracellular domains of TCRs or class II MHC molecules to associate. Thus, even if, *arguendo*, one of ordinary skill <u>had</u> been motivated to modify Dal Porto's complex to make a divalent TCR/IgG or class II MHC/IgG molecule, the

logical modification would have been to substitute one of the TCR or class II MHC extracellular domains for the MHC class I $\alpha$ chain in the fusion protein, to express the other extracellular domain by itself, analogous to Dal Porto's $\beta_2$ subunit, and to permit the two extracellular domains to associate as the prior art taught they would.

But this modification would not have formed the recited molecular complexes. To form the recited molecular complexes, the second extracellular domain must be fused to the immunoglobulin light chain. None of the cited prior art teaches or suggests associating the extracellular domains of a TCR or MHC class II molecule by fusing the domains to an immunoglobulin heavy and light chain. Moreover, none of the cited prior art teaches or suggests binding such a molecular complex to the surface of a cell.

Properly considered in their entireties, the combination of Matsui, Chang, Harris, and Dal Porto do not make the recited molecular complexes *prima facie* obvious. Matsui does not suggest construction of any molecules with higher binding affinities. Harris teaches away from using immunoglobulin heavy and light chains, which the recited molecular complexes contain. Chang teaches use of leucine zipper components to associate extracellular TCR domains, but the recited molecular complexes employ $\beta$ pleated sheets, not a leucine zipper. Dal Porto teaches a molecule with a substantially different structure. Each of Matsui, Chang, Harris, and Dal Porto teaches <u>soluble</u> molecules. The rejection does not make any specific factual findings to support the notion that one of ordinary skill would have been motivated to combine the cited references, much less to make the extensive modifications necessary to make the present invention.

Claims 28-32 and 51-55 are not *prima facie* obvious over the applied combination of Matsui, Chang, Harris, and Dal Porto. The Board should reverse the rejection.

## CONCLUSION

Neither the rejection of claims 32 and 56-58 under 35 U.S.C. § 112 ¶ 1 nor the rejection of claims 28-32 and 51-55 under 35 U.S.C. § 103(a) is legally correct. The Board should therefore reverse the rejections.

Respectfully submitted,

BANNER & WITCOFF, LTD.

/Lisa M. Hemmendinger/

Date: Monday, November 6, 2006 By: ______________________

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## APPENDIX 1. APPEALED CLAIMS

28. A composition comprising a cell in which a molecular complex is bound to the surface of the cell, wherein the molecular complex comprises at least two first fusion proteins and at least two second fusion proteins, wherein:

(a) each of the two first fusion proteins comprises an immunoglobulin heavy chain, wherein the immunoglobulin heavy chain comprises a variable region, and an extracellular portion of a first transmembrane polypeptide; and

(b) each of the two second fusion proteins comprises an immunoglobulin light chain and an extracellular portion of a second transmembrane polypeptide;

wherein the at least two first fusion proteins and the at least two second fusion proteins associate to form the molecular complex, wherein the molecular complex comprises two ligand binding sites, wherein each ligand binding site is formed by the extracellular domain of a first transmembrane polypeptide and the extracellular domain of a second transmembrane polypeptide, wherein the affinity of the molecular complex for a cognate ligand is increased at least two-fold over a dimeric molecular complex consisting of the first and the second fusion protein.

29. The composition of claim 28 wherein the first transmembrane polypeptide is an MHC class II$\beta$ chain and wherein the second transmembrane polypeptide is an MHC class II$\alpha$ chain.

30. The composition of claim 28 wherein the first transmembrane polypeptide is a TCR $\alpha$ chain and wherein the second transmembrane polypeptide is a TCR $\beta$ chain.

31. The composition of claim 28 further comprising a pharmaceutically acceptable carrier.

32. The composition of claim 28 wherein a population of the molecular complexes is bound to the cell, wherein an identical antigenic peptide is bound to each ligand binding site.

51. The composition of claim 28 wherein the immunoglobulin heavy chain is an IgG1 heavy chain.

52. The composition of claim 28 wherein the immunoglobulin light chain is an Igκ chain.

53. The composition of claim 28 wherein the first fusion proteins comprise a first peptide linker between the immunoglobulin heavy chain and the extracellular domain of the first transmembrane polypeptide and wherein the second fusion proteins comprise a second peptide linker between the immunoglobulin light chain and the extracellular domain of the second transmembrane polypeptide.

54. The composition of claim 53 wherein the first peptide linker is GLY-GLY-GLY-THR-SER-GLY (SEQ ID NO:10).

55. The composition of claim 53 wherein the second peptide linker is GLY-SER-LEU-GLY-GLY-SER (SEQ ID NO:11).

56. The composition of claim 32 wherein the antigenic peptides are bound to the ligand binding sites by a method comprising the step of:

incubating the cell in the presence of the antigenic peptides, whereby the antigenic peptides are bound to the ligand binding sites.

57. The composition of claim 32 wherein the antigenic peptides are bound to the ligand binding sites by a method comprising the steps of:

(a) alkaline stripping of the molecular complex to provide an alkaline stripped molecular complex;

(b) neutralization of the alkaline stripped molecular complex to provide a neutralized molecular complex;

(c) incubation of the neutralized molecular complex in the presence of an excess of the antigenic peptides; and

(d) slow refolding of the neutralized molecular complex in the presence of the excess of the antigenic peptides.

58. The composition of claim 32 wherein the antigenic peptides are covalently bound.

## APPENDIX 2. EVIDENCE RELIED UPON

| EVIDENCE | LOCATION IN THE RECORD | ATTACHMENT |
|---|---|---|
| Pages 125-37 of Abbas *et al.*, Cellular and Molecular Immunology, 3rd ed., W.B. Saunders Company, Philadelphia, 1997) | included with the response filed April 19, 2004 | 1 |
| PubMed search results for "antigenic peptide" and "peptide antigen" | included with the response filed April 19, 2004 | 2 |
| abstract of Smith *et al.*, J Immunol. 1979 Oct;123(4):1715-20 | included with the response filed April 19, 2004 | 3 |
| bibliographic information for Akuzawa & Tsuchiya, *Arerugi 14*, 519-21, 1965 | included with the response filed April 19, 2004 | 4 |
| Pages 105-07, page 147, and Table 7-2 of Abbas *et al.*, Cellular and Molecular Immunology, 3rd ed., W.B. Saunders Company, Philadelphia, 1997) | included with the response filed March 8, 2005 | 5 |
| Pages 41-43 of Abbas *et al.*, Cellular and Molecular Immunology, 3rd ed., W.B. Saunders Company, Philadelphia, 1997) | included with the response filed January 11, 2006 | 6 |
| U.S. Patent 5,723,309 | discussed at pages 8-9 of the response filed January 11, 2006 | 7 |
| U.S. Patent 5,583,031 | discussed at pages 8-9 of the response filed January 11, 2006 | 8 |

## APPENDIX 3. RELATED PROCEEDINGS

None.

# ATTACHMENT 1

SAUNDERS TEXT AND REVIEW SERIES

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THIRD EDITION

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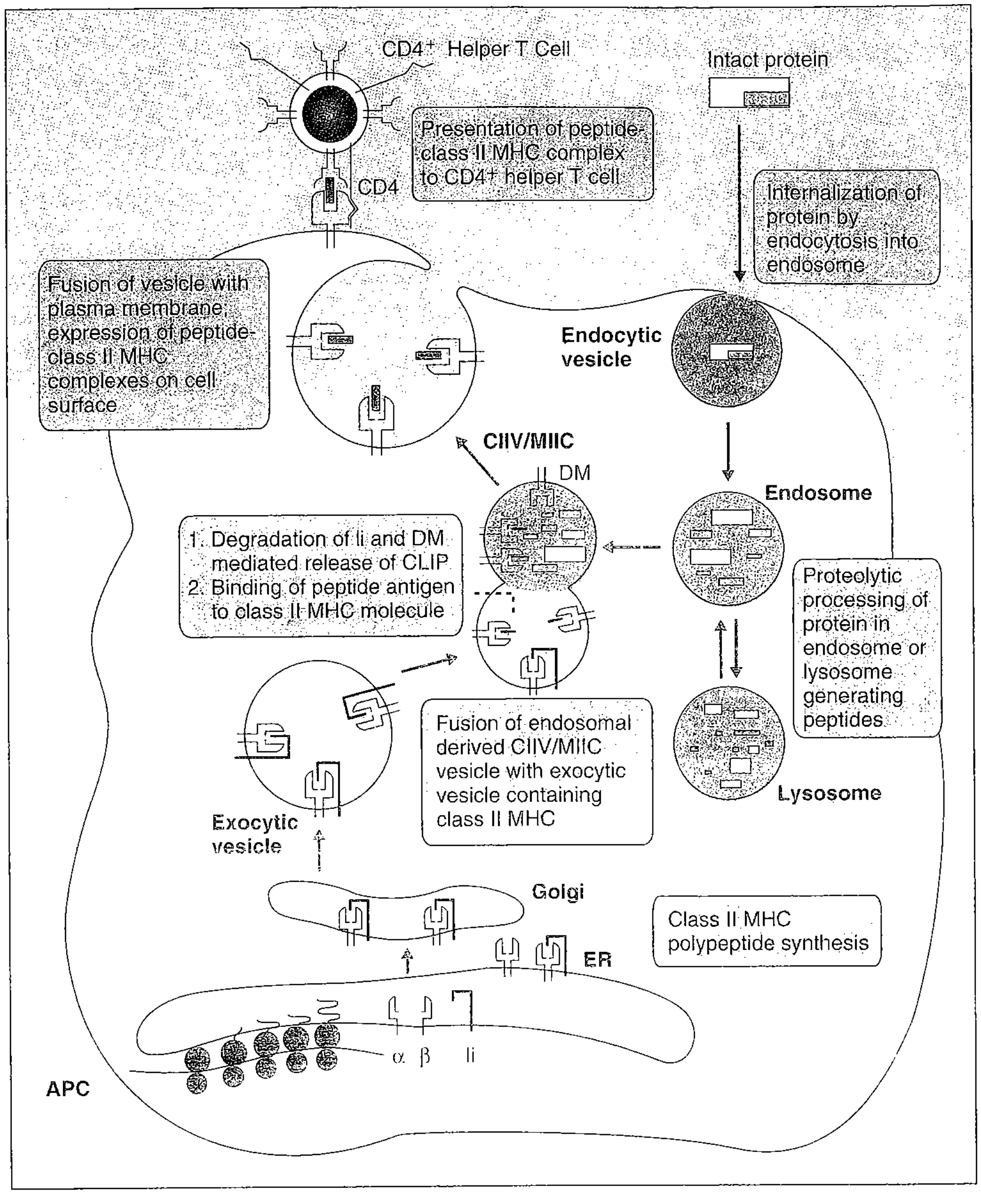


**FIGURE 6–5. The class II major histocompatibility complex (MHC) pathway of antigen presentation.** CLIP, class II–associated invariant chain peptide; Ii, invariant chain; ER, endoplasmic reticulum. Details of the functions of Ii and DM are shown in Figure 6–7.

that bind to class II MHC molecules, and most of these proteins are internalized from the extracellular environment. Thus, antigens made by extracellular bacteria, fungi, protozoa, and helminths are usually presented by the class II MHC pathway and activate CD4+ T cells. Additionally, some intact microorganisms can enter a cell by endocytosis or phagocytosis and survive within intracellular membrane-bound vesicles. Peptides derived from proteins made by these intracellular microorganisms may also be presented by class II MHC molecules.

## Processing of Internalized Proteins in Endosomal/Lysosomal Vesicles

The next step in antigen presentation is the processing of the antigen that was internalized in its native form. Several characteristics of the processing of extracellularly derived protein antigens are known:

1. *Antigen processing is a time- and metabolism-dependent phenomenon that takes place subsequent to internalization of antigen by APCs.* If macrophages (or other APCs) are incubated briefly ("pulsed") with a protein antigen such as ovalbumin, rendered metabolically inert by chemical fixation at various times thereafter, and tested for their ability to stimulate ovalbumin-specific T cells, functional antigen presentation occurs only if 1 to 3 hours elapse between the antigen pulse and fixation (Fig. 6–6). This time is required for the APCs to process the antigen and present it in association with class II MHC molecules on the cell surface. Processing of antigen is inhibited by maintaining the APCs below physiologic temperatures, by adding metabolic inhibitors such as azide, or by fixation earlier than 1 hour after the antigen pulse.

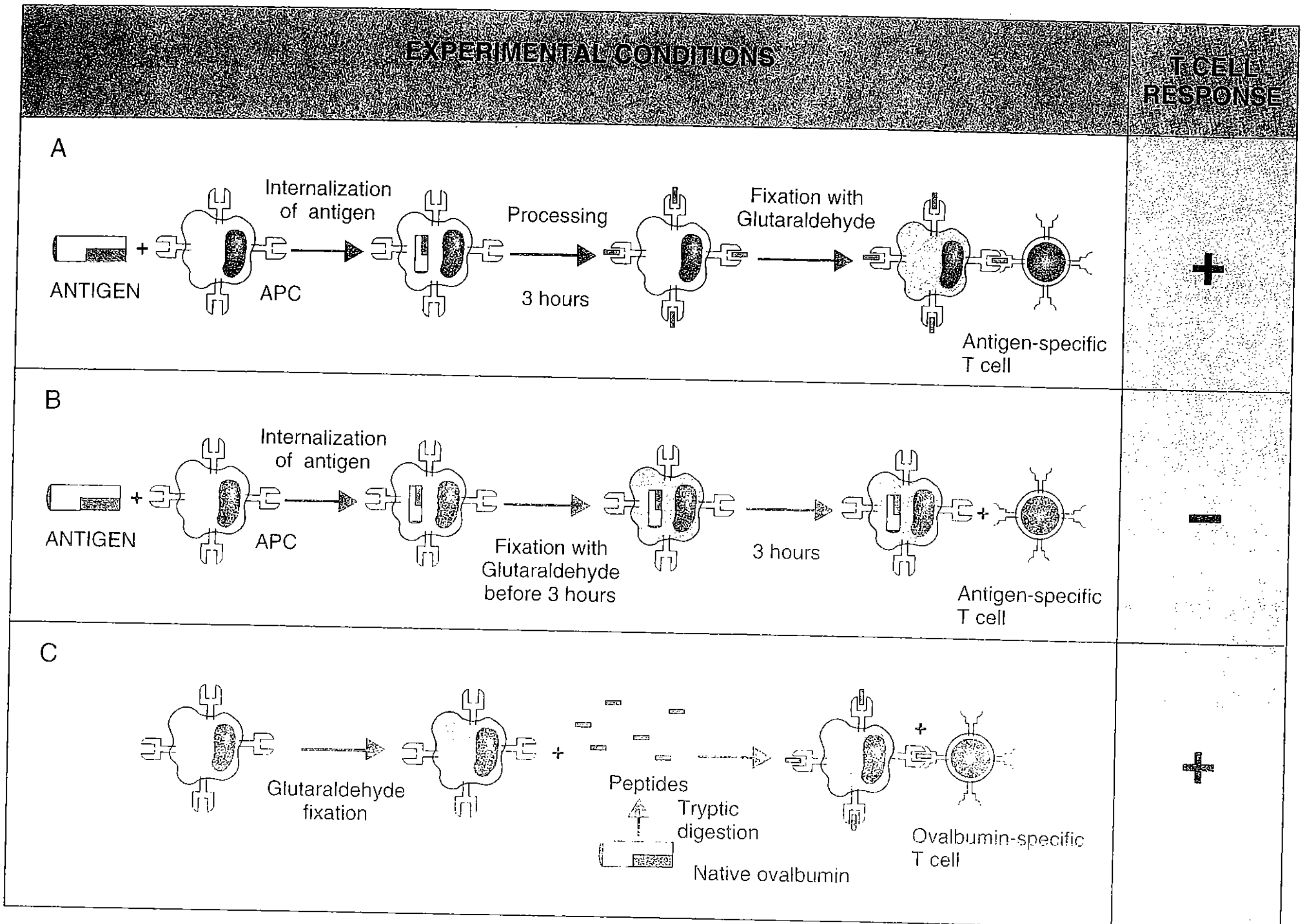


**FIGURE 6-6. Antigen processing requires time and cellular metabolism and can be mimicked by in vitro proteolysis.** If an antigen-presenting cell (APC) is allowed to process antigen and is then chemically fixed (rendered metabolically inert) 3 hours or more after antigen internalization, it is capable of presenting antigen to T cells (A). Antigen is not processed or presented if APCs are fixed less than 1 to 3 hours after antigen uptake (B). Fixed APCs bind and present proteolytic fragments of antigens to specific T cells (C). The artificial proteolysis, therefore, mimics physiologic antigen processing by APCs. Effective antigen presentation is assayed by measuring a T cell response, such as cytokine secretion. (Note that T cell hybridomas respond to processed antigens on fixed APCs, but growth factor–dependent T cells may require costimulators that are destroyed by fixation.)

2. *The endosomes and lysosomes where antigen processing takes place have an acidic pH, which is required for the processing.* Chemical agents that increase the pH of intracellular acid vesicles, such as chloroquine and ammonium chloride, are potent inhibitors of antigen processing.

3. *Cellular proteases are required for the processing of many protein antigens.* Several types of proteases, including cathepsin and leupeptin, are present in endosomes and lysosomes, and specific inhibitors of these enzymes block the presentation of protein antigens by APCs. The function of proteases is to cleave native protein antigens into small peptides. These proteases also probably act on the invariant chain, promoting its dissociation from class II MHC molecules, as discussed later. Most of these proteases function optimally at acid pH, and this is the likely reason why antigen processing occurs best in acidic compartments.

The processed forms of most protein antigens that T cells recognize can be artificially generated by proteolysis in the test tube. Macrophages that are fixed or that are treated with chloroquine before exposure to antigen can effectively present pre-digested peptide fragments of that antigen, but not the intact protein, to specific T cells (Fig. 6–6). Peptides that bind to MHC molecules and stimulate T cells can be analyzed for amino acid sequence and secondary structure to determine the nature of the potential ligands for T cell antigen receptors. Immunogenic peptides derived from many complex globular proteins, such as cytochrome c, ovalbumin, myoglobin, and lysozyme, have been characterized in detail in this way. More recently, naturally generated peptides have been eluted from the class II MHC molecules of APCs and analyzed for common structural characteristics. The physicochemical features of peptides that permit their binding to MHC molecules were described in Chapter 5.

The net result of processing of a protein antigen is the generation of peptides, many of which are 10 to 30 amino acids long and capable of bind-

ing to the peptide-binding clefts of class II MHC molecules. The requirement for antigen processing prior to T cell stimulation explains why T cells recognize linear but not conformational determinants of proteins and why T cells cannot distinguish between native and denatured forms of a protein antigen (see Table 6–1). It is likely that most types of APCs, including macrophages, B cells, and dendritic cells, are qualitatively similar in their ability to process endocytosed antigens; however, there may be quantitative differences. For instance, macrophages contain many more proteases than do B cells and are more actively phagocytic, so that macrophages may be more efficient than B cells at internalizing and processing large particulate antigens and presenting peptide fragments of these antigens. It is also possible that different APCs generate distinct sets of peptides from the same native protein because of differences in their endosomal proteases. Furthermore, different APCs may present different peptides because the set of class II MHC molecules expressed by one APC may not be identical to those expressed by another. Therefore, it is possible that the APCs involved in presenting a particular protein antigen can influence which T cells are activated by that antigen.

## Association of Processed Peptides With Newly Synthesized Class II MHC Molecules

Peptides generated by proteolysis of proteins in endosomes and lysosomes bind to newly synthesized class II MHC molecules within intracellular vesicles (see Fig. 6–5). The exact site of this association is not definitely known, but a variety of experimental data indicate that it occurs within an organelle of the endocytic pathway. An understanding of how peptide–class II MHC complexes are formed requires knowledge of the biosynthesis and subcellular transport of new class II MHC molecules. Several steps and key features of this process have been defined.

1. *The $\alpha$ and $\beta$ chains of class II MHC molecules are coordinately synthesized and associate with each other in the endoplasmic reticulum (ER).* These chains are translated from messenger ribonucleic acid (mRNA) molecules on membrane-bound ribosomes and are co-translationally inserted into the membrane of the ER.

2. *Newly synthesized class II heterodimers temporarily associate with two other nonpolymorphic polypeptides, not encoded by the MHC, which are required for proper assembly and transport of the MHC molecule.* The first of these proteins is called **calnexin** and it functions as a molecular chaperone, ensuring that the $\alpha$ and $\beta$ chains are properly folded during assembly of a class II MHC molecule. Calnexin is also involved in the assembly of other multichain molecules in the ER, including class I MHC molecules and the T cell antigen receptor (see Chapter 7). The second nonpolymorphic protein associated with the class II MHC $\alpha\beta$ heterodimers in the ER is called the **invariant chain (Ii).** This protein is a 30 kD Ig superfamily member which is a type II membrane protein, i.e., it has a reverse orientation to most transmembrane proteins, so that the amino terminus is intracytoplasmic and the carboxy terminus is intraluminal. The native invariant chain is a homotrimer. Each subunit binds one newly synthesized class II $\alpha\beta$ heterodimer, forming a nine polypeptide chain complex (i.e., three $\alpha\beta$ heterodimers bound to one invariant chain homotrimer). Only after the invariant chain binds the $\alpha\beta$ heterodimer is calnexin released, and the class II–invariant chain complex is able to move out of the ER.

3. *The invariant chain prevents peptides or nascent unfolded polypeptides in the ER from binding to newly formed class II MHC $\alpha\beta$ heterodimers.* The invariant chain binds to the class II MHC heterodimer in a way that interferes with peptide loading of the cleft formed by the $\alpha$ and $\beta$ chains. There are, in fact, peptides within the ER derived from cytosolic proteins, as we will discuss later. Since the effector functions of class II–restricted T cells are best suited for dealing with extracellular microbes, it would be counterproductive to have class II MHC molecules loaded with peptides derived from cytosolic proteins. Furthermore, since the peptide binding cleft of class II MHC molecules has open ends, it can theoretically accommodate binding of newly translated polypeptides which have not yet folded into their tertiary structural conformation. Such polypeptides are abundant in the ER, but the presence of the invariant chain prevents their association with class II MHC molecules.

4. *The invariant chain also directs newly formed class II MHC molecules to specialized endosomal/lysosomal organelles where internalized proteins are proteolytically degraded into peptides.* In the ER, N-linked oligosaccharides are added to the newly translated class II MHC $\alpha$ and $\beta$ chains, the two chains form heterodimers, and the heterodimers associate with invariant chains. Subsequent to these events, the class II MHC–invariant chain complexes pass through the Golgi apparatus, where the oligosaccharides are further modified. Then the invariant chain targets the movement of the mature class II MHC molecules to specialized membrane-bound organelles of the endocytic pathway that contain proteolytically degraded proteins derived from the extracellular milieu. The invariant chain performs this function by virtue of certain amino acid sequences in its amino terminal cytoplasmic tail. Immunoelectronmicroscopy and subcellular fractionation studies have been used to define specific characteristics of this subcellular compartment targeted by the invariant chain. In macrophages, it is called the MHC class II compartment or MIIC and has the properties of a vesicle in transition between endosome and lysosome, in-

cluding high density and a characteristic multivesiculated appearance. In some B cells, a similar but less dense organelle containing invariant chain and class II MHC has been identified and named the class II vesicle (CIIV). These organelles likely represent specialized branch points in the vesicular transport pathways that allow newly formed class II MHC molecules on their way to the cell surface to become exposed to endocytically derived peptides. Thus, *the invariant chain plays a key role in getting MHC molecules to the same place as peptides derived from extracellular protein antigens.*

5. *Within the MIIC/CIIV compartment, the invariant chain is removed from class II MHC molecules by the combined action of proteolytic enzymes and the HLA-DM molecule* (see Fig. 6–7). Since the invariant chain blocks access to the peptide-binding groove of a class II MHC molecule, it must be removed before complexes of peptide and class II MHC can form. The same proteolytic enzymes that generate peptides from internalized proteins also act on the invariant chain in a stepwise fashion, leaving only a 24 amino acid remnant called **c**lass II–associated **i**nvariant chain **p**eptide **(CLIP)**. X-ray crystallographic analysis has shown that the CLIP peptide sits in the peptide-binding cleft in the same way that other peptides bind to class II MHC molecules. Therefore, removal of CLIP is required before access is provided to peptides from extracellular proteins. This is accomplished by the action of a molecule called HLA-DM (or H-2M in the mouse), which is encoded within the MHC and has a structure very similar to that of class II MHC molecules. HLA-DM molecules differ from class II MHC molecules in several respects: they are not polymorphic, they do not necessarily associate with invariant chain, they are not expressed on the cell surface, and their subcellular distribution is distinct from class II MHC molecules. Nonetheless, HLA-DM is found in the MIIC compartment. Mutant cell lines which lack DM are defective in presenting peptides from extracellularly derived proteins. When class II MHC molecules are isolated from these DM-mutant cell lines, they are found to have almost exclusively CLIP peptides in their peptide-binding clefts, consistent with a role for DM in removing CLIP. *In vitro* studies have confirmed that HLA-DM acts as a peptide exchange molecule, facilitating the removal of CLIP and the addition of other peptides to class II MHC molecules. Predictably, DM gene knockout mice have profound defects in class II MHC–restricted antigen presentation.

6. *Once CLIP peptides are removed, peptides generated by proteolytic cleavage of extracellularly derived protein antigens bind to class II MHC mole-*

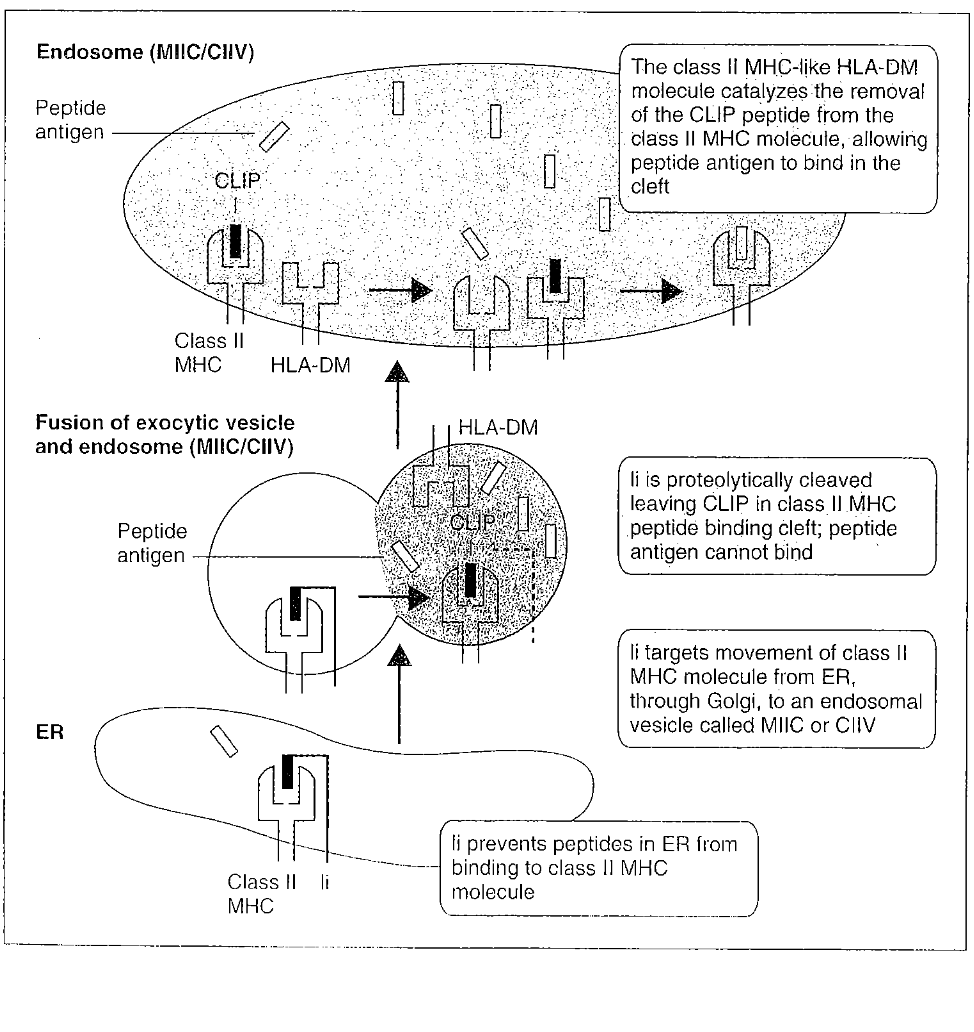


**FIGURE 6–7. The functions of class II major histocompatibility complex (MHC)–associated invariant chains and HLA-DM.** ER, endoplasmic reticulum; Ii, invariant chain.

cluding high density and a characteristic multivesiculated appearance. In some B cells, a similar but less dense organelle containing invariant chain and class II MHC has been identified and named the class II vesicle (CIIV). These organelles likely represent specialized branch points in the vesicular transport pathways that allow newly formed class II MHC molecules on their way to the cell surface to become exposed to endocytically derived peptides. Thus, *the invariant chain plays a key role in getting MHC molecules to the same place as peptides derived from extracellular protein antigens.*

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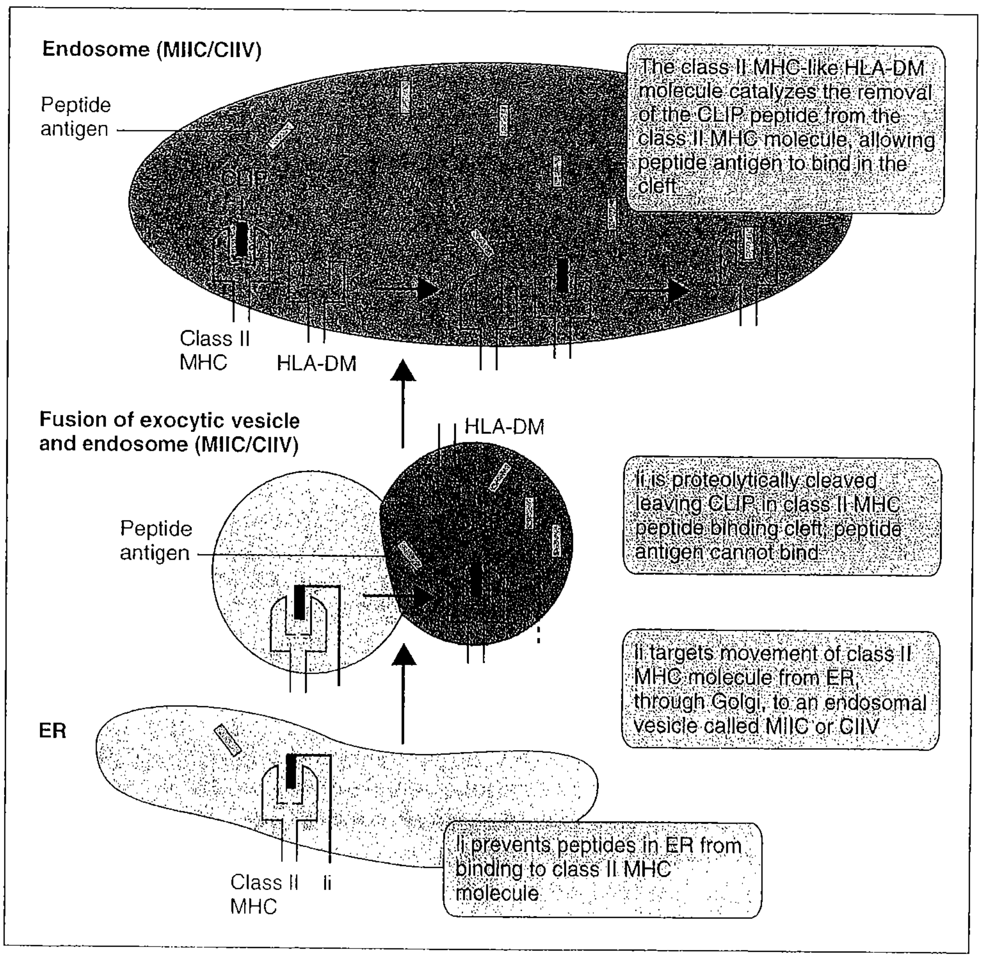


**FIGURE 6–7. The functions of class II major histocompatibility complex (MHC)–associated invariant chains and HLA-DM.** ER, endoplasmic reticulum; Ii, invariant chain.

*cules*. Although initial studies of the physical interaction of peptides with class II MHC molecules indicated a very slow association rate, requiring up to 48 hours to achieve saturation, more recent analyses indicate that HLA-DM greatly enhances this process, so that peptides can form stable complexes with class II MHC molecules within 20 minutes. Since the ends of the class II MHC peptide-binding cleft are open, large peptides or even unfolded whole proteins may bind, yet the size of peptides eluted from cell surface class II MHC molecules is restricted to between 10 and 30 amino acids. It is possible, therefore, that proteolytic enzymes "trim" bound polypeptides to the appropriate size for T cell recognition after the polypeptides bind to class II MHC molecules.

7. *Peptide binding to class II MHC molecules stabilizes the $\alpha\beta$ heterodimer, and the peptide dissociation rate is extremely slow.* The ability of peptide to increase the tightness of association of the class II MHC $\alpha$ and $\beta$ chains serves to increase the likelihood that only properly loaded peptide-MHC complexes will survive long enough to get displayed on the cell surface. A similar phenomenon occurs in class I MHC assembly. The long life of a peptide-MHC complex increases the chance that a T cell specific for such a complex will make contact, bind, and be activated by that complex.

8. *Stable peptide-class II MHC complexes are delivered to the cell surface by membrane fusion with exocytic vesicles, and they are displayed there for surveillance by CD4⁺ T cells.*

Only a very small fraction of cell surface peptide-MHC complexes will contain the same peptide. Furthermore, most of the bound peptides will be derived from normal self proteins, since there is no mechanism to distinguish self from foreign proteins in the process that generates the peptide-MHC complexes. This has been demonstrated by amino acid analysis of peptides eluted from class II MHC molecules purified from B cells grown in tissue culture. Most of these peptides were derived from self proteins. These findings raise two important questions. First, if individuals process their own proteins and present them in association with their own class II MHC molecules, why do we normally not develop immune responses against self proteins? It is likely that self-tolerance is mainly due to the absence or inactivation of T cells capable of recognizing and responding to self antigens, and this is why self peptide-MHC complexes do not normally induce autoimmunity (see Chapters 8 and 19). Second, how can a T cell recognize and be activated by specific foreign antigen when it encounters an APC surface that is predominantly displaying self-peptide–MHC complexes? The answer lies in part with the extraordinary sensitivity of T cells for specific peptide-MHC complexes. It has been estimated that as few as 100 to 200 complexes of a particular peptide with a particular allelic form of class II MHC molecule on the surface of an APC can lead to activation of a T cell. This represents less than 0.1 per cent of the total number of class II molecules likely to be present on the surface of the APC, most of which would be occupied with self peptides. In fact, the indiscriminate ability of the APC to internalize, process, and present the heterogeneous mix of self and foreign extracellular proteins ensures that the immune system will not miss transient or quantitatively small exposures to foreign antigens. Furthermore, there is evidence that a single T cell will sequentially engage multiple peptide-MHC complexes until achieving a sufficient threshold of activating signals (see Chapter 7).

Although the bulk of experimental evidence supports the model described above for the generation of most class II MHC–peptide complexes, there are potentially important alternate intracellular pathways for the generation of these complexes that may be immunologically significant. First, it is possible that cell surface class II molecules may be recycled by internalization into endosomes, where they bind newly generated peptide fragments of internalized protein. This process would likely require an exchange of previously bound peptides with the new ones. Second, there are exceptions to the general case that class II MHC molecules bind peptides derived from internalized exogenous proteins. Cell surface complexes of class II MHC molecules with peptides derived from endogenously synthesized proteins have been detected both by T cell responses to such proteins and by direct analysis of eluted peptides from cell surface–derived class II MHC molecules. In some cases, this may result from a normal cellular pathway for the turnover of cytoplasmic contents, referred to as autophagy. In this pathway, cytoplasmic contents are entrapped within ER-derived membrane vesicles called autophagosomes, these vesicles fuse with lysosomes, and the cytoplasmic proteins are proteolytically degraded. The association of the peptides generated by this route would require movement of the peptides to a class II–bearing vesicular compartment, as described previously for trafficking of exogenously derived peptides. In addition, some peptides that associate with class II MHC are derived from endogenously synthesized membrane proteins. Before they are expressed on the surface, these proteins may have ready access to class II MHC molecules because they would be synthesized and transported through the same ER-Golgi compartments as the membrane-bound class II MHC molecules themselves. How such membrane proteins are processed is currently unknown. It is also possible that after cell surface expression, membrane proteins may reenter the cell by the same endocytic pathway as exogenous proteins. In this way, peptides derived from virally encoded membrane proteins may enter the class II–MHC pathway of antigen presentation. This is a theoretically important way in which viral antigen-specific CD4⁺ helper T cells may be activated.

## MECHANISMS OF ANTIGEN PRESENTATION TO CLASS I MHC–RESTRICTED CD8+ T CELLS

As we have mentioned previously, CD8+ T cells, most of which are CTLs, recognize peptides that are usually derived from protein antigens that are synthesized within APCs, processed, and subsequently expressed on the APC surface in association with class I MHC molecules. Examples of endogenously synthesized foreign proteins are viral proteins and the products of mutated or dysregulated genes in tumor cells. CTLs are the principal immunologic defense mechanisms against viruses and may be important in the immune destruction of tumors. In contrast to the restricted expression of class II MHC molecules, almost all cells express class I MHC molecules and have the ability to display peptide antigens in association with these MHC molecules on the cell surface. This ensures that any cell synthesizing viral or mutant proteins can be marked for recognition and killing by CD8+ CTLs. As is the case with class II MHC–associated antigen presentation, generation of peptide–class I MHC complexes is a continuous normal function of cells, which does not discriminate between foreign and self proteins. This portion of the chapter describes the known features of the generation of peptide–class I MHC complexes on the surface of cells. The principal steps in this pathway are as follows (Fig. 6–8):

1. Synthesis of protein antigens in the cytosol or delivery of protein antigens into the cytosol
2. Proteolytic degradation of cytosolic proteins into peptides
3. Transport of peptides into the ER
4. Assembly of peptide–class I MHC complexes within the ER
5. Expression of peptide–class I MHC complexes on the cell surface

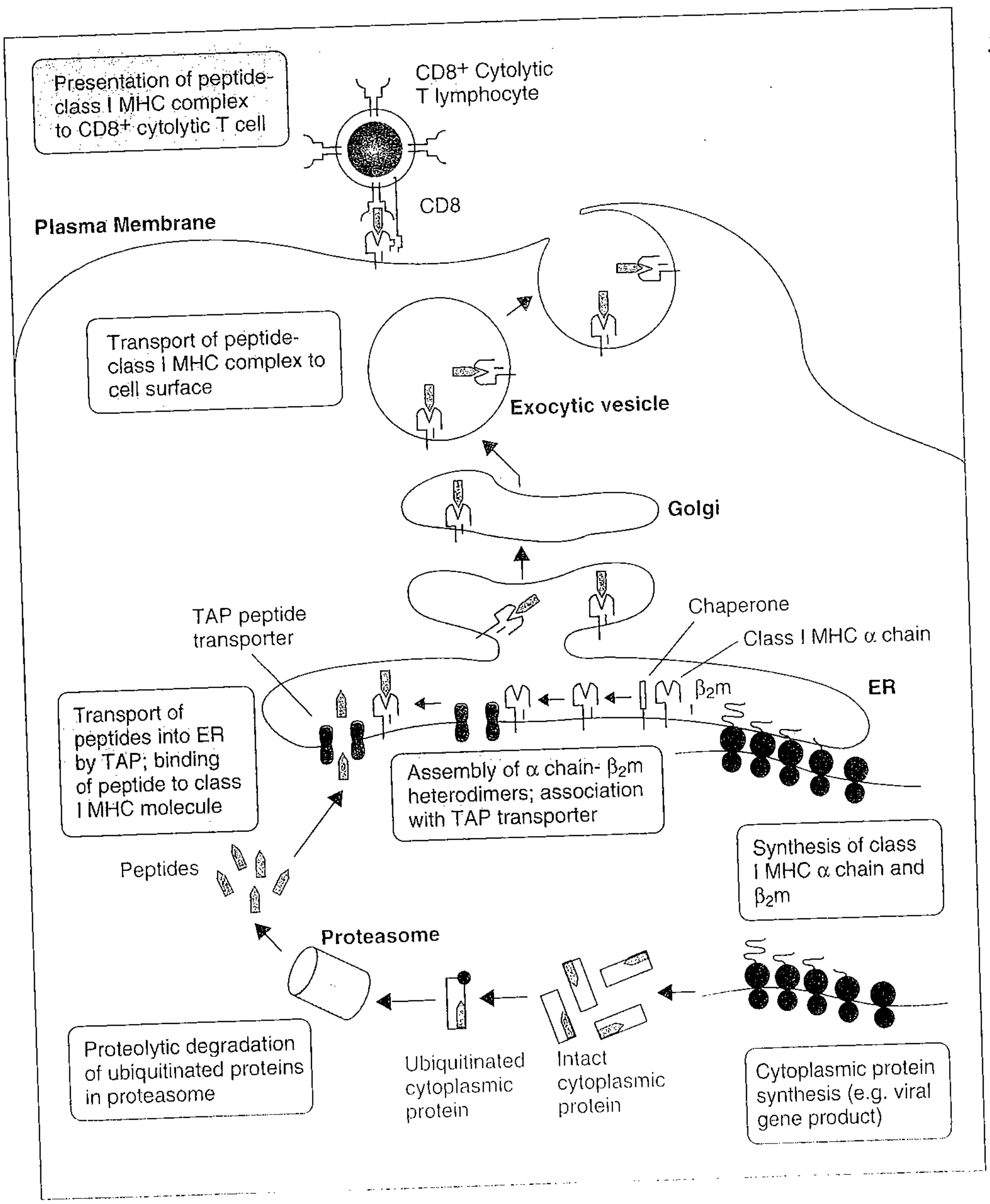


**FIGURE 6–8. The class I major histocompatibility complex (MHC) pathway of antigen presentation.** TAP, transporter associated with antigen presentation; ER, endoplasmic reticulum; $\beta_1$m, $\beta_2$ microglobulin.

## Entry of Cytosolic Proteins Into the Class I–MHC Pathway of Antigen Presentation

*The prerequisite for entry of a protein into the processing pathway leading to peptide–class I MHC association is simply location in the cytosol.* Several lines of evidence support this.

1. If a viral protein, such as influenza nucleoprotein, or a protein like ovalbumin, is added in soluble form to a cell that expresses class I and class II MHC molecules, the antigen is internalized, processed, and presented only in association with class II MHC molecules. Such exogenously added antigens will be recognized by class II–restricted, antigen-specific CD4+ T cells but will not sensitize the APC to lysis by CD8+ T cells. On the other hand, if the gene encoding the viral protein or ovalbumin is transfected into the APCs so that the antigen is synthesized on polyribosomes in the cytosol, the cell becomes sensitive to lysis by specific class I–restricted CD8+ T cells (see Fig. 6–3).
2. If an antigen is introduced into the cytoplasm of a cell by making the plasma membrane transiently permeable to macromolecules or by membrane fusion of an APC with lipid vesicles containing the protein, the antigen is subsequently processed and peptides associate only with class I MHC molecules (see Fig. 6–3).

The significance of having cytosolic proteins enter the class I MHC pathway of antigen presentation lies in the fact that endogenously synthesized foreign or mutant proteins will be present in the cytosol, and therefore will target cells for lysis by CD8+ class I MHC–restricted CTL. For example, viruses encode RNA transcripts, which are translated into foreign proteins in the host cell cytoplasm. Therefore, peptides derived from viral protein antigens end up being displayed on class I MHC molecules on the surface of virally infected cells. This enables class I MHC–restricted CD8+ cytolytic T cells to recognize the virally infected cells and destroy them. Since virtually all nucleated cells express class I MHC, any virus-infected cell is susceptible to CTL-mediated lysis. Similarly, CTLs may be important in recognizing and killing cancer cells, which often express mutated genes or unmutated genes that are not expressed in normal adult cells (see Chapter 18). The products of such endogenous genes may be expressed in the cytosol. In addition, some intracellular microbes, such as mycobacteria, reside for long periods of time within phagocytic vesicles. It is possible that there will be some breakdown in the membrane barrier of these vesicles, resulting in the microbial proteins leaking into the cytoplasm, and thus gaining access to the class I MHC pathway of antigen presentation. Alternatively, there may be specific transport mechanisms that deliver proteins or peptides from these vesicles to the cytoplasm.

## Processing of Cytosolic Antigens

The intracellular mechanisms that generate antigenic peptides which bind to class I MHC molecules are very different from the mechanisms described earlier for peptide–class II MHC molecule associations. This is evident from the observations that the agents that raise endosomal and lysosomal pH, or directly inhibit endosomal proteases, block class II– but not class I–associated antigen presentation.

*Peptides that bind to class I MHC molecules are proteolytically generated in the cytoplasm* prior to entry into the exocytic pathway that delivers the peptide–MHC protein complex to the cell surface. This conclusion is supported by a variety of experimental observations.

1. A cell infected with a virus becomes sensitized to lysis by virus-specific CTLs; this is because the cell displays peptides derived from viral proteins in association with class I MHC molecules on the cell surface. Some of these proteins, such as influenza nucleoprotein, are neither membrane bound nor secreted, i.e., they do not gain access to exocytic pathways in their intact form. Furthermore, the genes encoding viral membrane proteins can be altered to eliminate the membrane insertion sequences. When these genes are transfected into cells, the encoded proteins cannot gain access to the ER and exocytic pathway, yet peptides from these proteins are still presented to CD8+ CTLs.
2. When peptide epitopes for CTL recognition are synthesized directly in the cytoplasm of a cell as products of transfected minigenes, the cell becomes sensitized for lysis. This implies that peptides generated in the cytoplasm have direct access to the exocytic pathway for cell surface expression of class I MHC molecules.

*A major mechanism for the generation of peptides from cytosolic protein antigens is proteolysis in the* ***proteasome,*** a large multiprotein complex with a broad range of proteolytic activity that is found in the cytoplasm of most cells. A 700 kD form of proteasome appears as a cylinder composed of a stacked array of four inner and four outer rings, with each ring composed of seven distinct subunits. The subunits of the inner rings are the catalytic sites for proteolysis. A larger, 1500 kD proteasome is likely to be most important *in vivo* and is composed of the 700 kD structure plus several additional subunits that regulate proteolytic activity. Two catalytic subunits present in many 1500 kD proteasomes, called LMP2 and LMP7, are encoded by genes in the MHC (see Chapter 5). Both LMP2 and LMP7 expression are upregulated by IFN-$\gamma$, leading to an increase in the number of proteasomes containing these subunits. The proteasome performs a basic housekeeping function in cells by degrading many different cytoplasmic proteins. For example, NF-$\kappa$B activation is dependent on proteasomal degradation of I$\kappa$B (see Box 4–4, Chap-

ter 4). Proteins are targeted for proteasomal degradation by covalent linkage of several copies of a small polypeptide called ubiquitin. This process of polyubiquitination requires adenosine triphosphate (ATP) and a variety of enzymes. Several lines of evidence suggest that the proteasome, and probably ubiquitination, are involved in antigen processing for the class I MHC pathway of antigen presentation.

1. In some experimental situations, inhibition of the enzymes required for ubiquitination also inhibits the presentation of cytoplasmic proteins to class I MHC–restricted T cells specific for a peptide epitope of that protein.
2. Modification of proteins by attachment of an N-terminal sequence which is recognized by ubiquitin-conjugating enzymes leads to enhanced ubiquitination and more rapid class I MHC–associated presentation of peptides derived from those proteins.
3. Specific inhibitors of proteasomal function, such as peptide aldehydes, block presentation of a cytoplasmic protein to class I MHC–restricted T cells specific for a peptide epitope of that protein.
4. Proteasomes typically generate peptides between five and 11 amino acids long, which includes the lengths that best fit the peptide-binding clefts of class I MHC molecules.
5. The specificity of proteolysis by LMP-2– and LMP-7–containing proteasomes from IFN-$\gamma$–treated cells favors the generation of peptides with C-terminal basic or hydrophobic amino acid residues, which are typical of many class I MHC–binding peptides.

There are many examples of protein antigens that apparently do not require ubiquitination or proteasomes in order to be presented by the class I MHC pathway. In some cases this may reflect the fact that other, less well-defined mechanisms of cytoplasmic proteolysis exist. In addition, some class I MHC–binding peptides may be generated by proteolytic enzymes resident in the ER. For example, peptides from secretory proteins with hydrophobic signal sequences are often found associated with class I MHC molecules. These proteins are targeted directly to the ER during translation and therefore may bypass cytoplasmic degradation.

## Delivery of Peptides From Cytoplasm to the ER

Class I MHC molecules are assembled in the ER, and this process is dependent on peptides. Since peptides generated in the cytosol are presented by class I MHC molecules, a mechanism must exist for delivery of cytosolic peptides into the ER. The initial insights into this mechanism came from studies of cell lines that are defective in assembling and displaying peptide–class I MHC complexes on their surfaces. The mutations responsible for this defect turned out to involve two genes in the MHC, which are homologous to a family of genes that encode proteins that mediate ATP-dependent transport of low molecular weight compounds across intracellular membranes. The two genes in the MHC that belong to this family encode proteins called transporter associated with antigen presentation-1 or **TAP-1,** and **TAP-2.** TAP-1 and TAP-2 form heterodimers, which are localized in the ER and *cis*-Golgi (Fig. 6–8). In this location they mediate the active, ATP-dependent transport of peptides from the cytosol into the ER lumen. Although the TAP heterodimer has a broad range of specificities, it optimally transports peptides ranging from eight to 12 amino acid residues long and therefore delivers to the ER peptides of the right size for binding to class I MHC molecules. Mice with targeted disruptions of the genes encoding TAP-1 or TAP-2 show defects in class I MHC expression and cannot effectively present proteins to class I MHC–restricted T cells. Rare examples of human TAP-2 gene mutations have also been identified, and predictably, the patients carrying these mutant genes also show defective class I MHC–associated antigen presentation.

## Assembly and Surface Expression of Peptide–Class I MHC Complexes

The actual assembly and surface expression of stable class I MHC molecules require the presence of peptides. A variety of experimental data have indicated a particular sequence of events in assembly and expression of peptide–class I MHC complexes:

1. The class I MHC $\alpha$ chain and $\beta_2$ microglobulin are synthesized on the rough ER and transported into the smooth ER as separate polypeptide chains.
2. The $\alpha$ chain associates with molecular chaperones, which prevent degradation and promote proper folding of the protein. Two chaperones that are known to associate with the $\alpha$ chain in the ER are BiP, a member of the heat shock protein family, and calnexin.
3. $\beta_2$ microglobulin binds to partially or completely folded $\alpha$ chain and the chaperones dissociate. These newly formed $\alpha$ chain–$\beta_2$ microglobulin dimers are unstable and cannot be transported efficiently out of the ER.
4. The $\alpha$ chain–$\beta_2$ microglobulin dimers move to and become physically associated with the luminal aspects of the TAP proteins within the ER. This close association ensures that peptides transported into the ER by the TAP bind to the associated empty class I MHC molecules. It is also possible that the TAP association promotes further folding of the $\alpha$ chain and $\beta_2$ microglobulin.
5. Peptide binding to the class I molecule greatly enhances its stability and causes its release from the TAP protein.
6. Stable peptide–class I MHC complexes now move through the Golgi, where the MHC molecules

undergo further carbohydrate modification, and then they are transported to the cell surface by exocytic vesicles. Surface complexes can now be recognized by CD8+ T cells.

The requirement for peptides in class I MHC assembly has been clearly shown by analysis of TAP-deficient cells (either mutant cell lines or cells from TAP-1 gene knockout mice), which express significantly reduced levels of surface class I MHC (Fig. 6–9). Since TAP delivers peptides to the ER, these findings suggest that peptides in the ER are required for class I MHC assembly. Those class I MHC molecules that do get expressed in TAP-deficient cells have bound peptides that are mostly derived from signal sequences of proteins destined for secretion or membrane expression. These signal sequences are cleaved off and degraded to peptides within the ER during translation, without a requirement for TAP. There are two reasons why peptides transported into the ER preferentially bind to class I and not class II MHC molecules. First, as we have discussed, newly formed class I MHC molecules are bound to the luminal aspect of the TAP complex. Second, as mentioned previously, in the ER the class II MHC–peptide-binding cleft is blocked by the invariant chain.

The sequence of events in class I MHC molecule assembly which we have discussed ensures that only properly folded, peptide-loaded class I MHC molecules are displayed for T cell surveillance. A few empty class I MHC complexes do make it out to the cell surface, but these are unstable and rapidly dissociate. It is, of course, likely that there are other steps involved in this pathway that are not yet resolved, and it is also possible that alternate pathways may exist. Nonetheless, the effects of mutations and inhibitors of this pathway, as discussed, indicate that it is critical for normal immune function. Furthermore, the importance of this pathway to anti-viral immunity is demonstrated by the evolution of viral mechanisms that interfere with it. For example, herpes simplex virus produces a protein, called ICP47, that effectively plugs up the TAP pore through which peptides are delivered to the ER and thus prevents presentation of viral antigens to T cells (see Chapter 16).

## PHYSIOLOGIC SIGNIFICANCE OF MHC-ASSOCIATED ANTIGEN PRESENTATION

So far we have discussed the specificity of CD4+ and CD8+ T lymphocytes for MHC-associated foreign protein antigens and the mechanisms by which complexes of peptides and MHC molecules

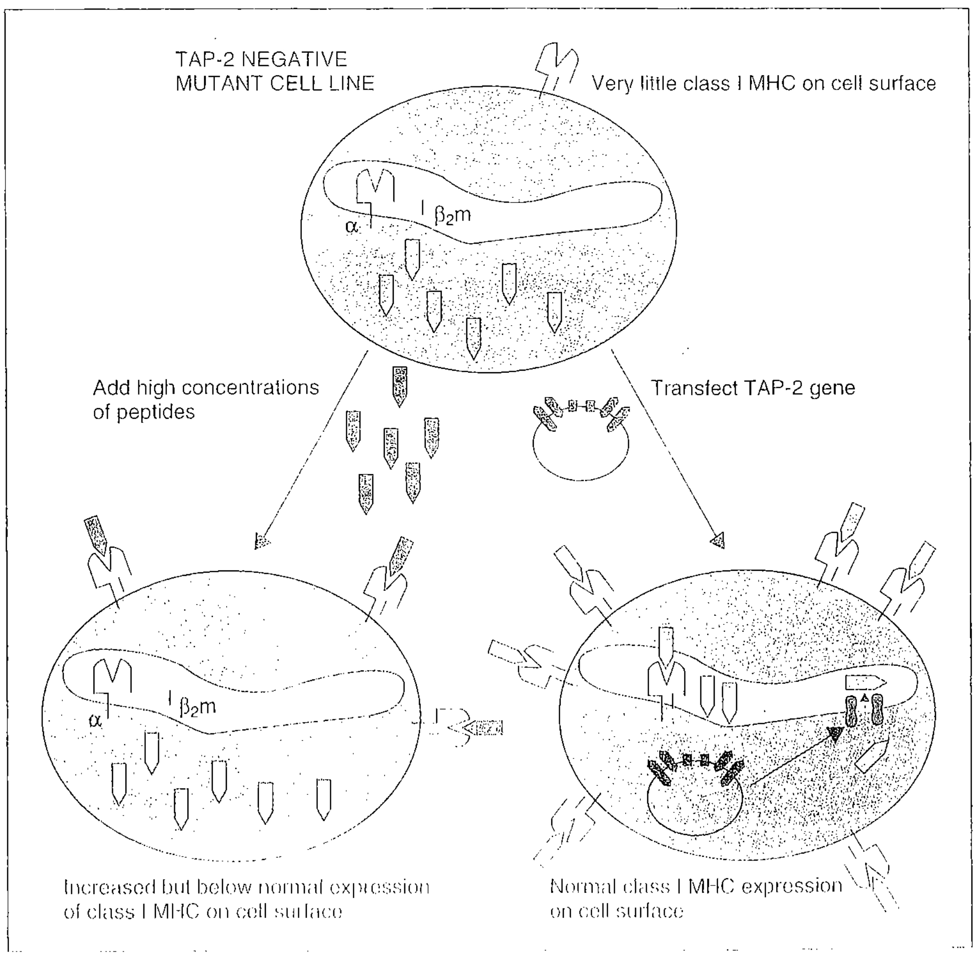


**FIGURE 6–9. TAP gene products are required for assembly and cell surface expression of peptide–class I major histocompatibility (MHC) complexes.** A cell line with a nonfunctional TAP-2 gene expresses very few surface class I MHC molecules. The peptides bound to these few surface class I MHC molecules are predominantly derived from the signal sequences of membrane or secreted proteins. The addition of high doses of peptides can induce some class I MHC molecule assembly and expression. In this case, it is not known whether the assembly of the peptide–class I complexes occurs at the cell surface or intracellularly. When a functional TAP-2 gene is transfected into the cell line, normal assembly and expression of peptide–class I MHC molecules are restored.

are produced. There are several fundamental properties of T cell–mediated immune responses that are consequences of the fact that T cells only recognize MHC-associated antigens. In this section, we will consider the impact of MHC-associated antigen presentation on the role that T cells play in protective immunity, the nature of T cell responses to different antigens, and the limitations of what T cells will recognize in protein antigens.

## T Cell Surveillance for Foreign Antigens

As we discussed throughout this chapter, *both the class I and class II MHC pathways of antigen presentation sample pools of predominantly normal self proteins for display to the T cell repertoire, which surveys these samples for the rare foreign or mutant peptide.* The recent advances in our understanding of how peptide-MHC complexes are formed confirm that MHC molecules are scaffolds for peptide display to the immune system and that antigen processing pathways have evolved to sample both extracellular and intracellular proteins in order to supply the peptides. The specialized class II MHC-expressing APCs have various characteristics, such as the phagocytic activity of macrophages, the high-affinity Ig antigen receptors on B cells, and the long cytoplasmic processes of dendritic cells, which enable them to encounter the full range of possible extracellular protein antigens. The convergence of the endocytic pathways in these cells with the exocytic pathway of class II MHC expression ensures that peptides derived from these extracellular antigens will be displayed on the cell surface for possible recognition by $CD4^+$ T cells. The widespread expression of class I MHC in nucleated cells, and the pathway of peptide loading of class I MHC molecules which is linked to a ubiquitous mechanism for degrading cellular proteins, ensures that peptides from virtually any intracellular protein will be displayed for possible recognition by $CD8^+$ T cells. Superimposed on this system of antigen presentation is a sensitive system of T cell surveillance of the displayed peptides, which is based on continuous recirculation of T cells to sites of APCs throughout the body, and the exquisite sensitivity of T cells, allowing them to respond to small numbers of peptide-MHC complexes. Thus, the paradox that antigen presentation mechanisms overwhelmingly display normal self peptides is actually fundamental to the ability of the immune system to find rare foreign protein antigens.

## The Nature of T Cell Responses

Based on our understanding of antigen presentation to T cells, we can now explain other physiologic consequences of MHC-restricted antigen recognition that were introduced in Chapter 5.

1. Because T cells recognize only MHC-associated peptide antigens, they can respond only to antigens associated with other cells (the APCs) and are unresponsive to soluble or circulating proteins. *This unique specificity for cell-bound antigens is essential for the functions of T lymphocytes, which are largely mediated by cell-cell interactions and by cytokines that act at short distances.* For instance, helper T cells activate B cells and macrophages. Not surprisingly, B lymphocytes and macrophages are two of the principal cell types that express class II MHC genes, function as APCs for $CD4^+$ helper T cells, and focus helper T cell effects to their immediate vicinity. Similarly, CTLs can lyse any nucleated cell producing a foreign antigen, and all nucleated cells express class I MHC molecules, which are the restricting elements for antigen recognition by $CD8^+$ CTLs.

2. The triaging of endosomal versus cytoplasmic proteins to class II or class I MHC pathways of antigen presentation determines which subsets of T cells will respond to antigens found in those two pools of proteins (Fig. 6–10). Extracellular antigens usually end up in the endosomal pool and activate class II–restricted $CD4^+$ T cells. These cells function as helpers to stimulate effector mechanisms such as antibodies and phagocytes that serve to eliminate extracellular antigens. Conversely, endogenously synthesized antigens are present in the cytoplasmic pool of proteins and usually activate class I–restricted $CD8^+$ CTLs. These lymphocytes lyse cells producing intracellular antigens. *Thus, antigens from microbes that reside in different locations selectively stimulate the T cell populations that are most effective at eliminating that type of microbe.*

## Immunogenicity of Protein Antigens

MHC molecules may determine the immunogenicity of protein antigens in two related ways:

1. *The immunodominant epitopes of complex proteins are often the peptides that bind most avidly to MHC molecules.* If an individual is immunized with a multideterminant protein antigen, in many instances the majority of the responding T cells are specific for one or a few linear amino acid sequences of the antigen. These are called the "immunodominant" determinants or epitopes. For instance, in $H\text{-}2^k$ mice immunized with hen egg lysozyme (HEL), more than half the HEL-specific T cells are specific for the epitope formed by residues 46-61 of HEL in association with the $I\text{-}A^k$ but not the $I\text{-}E^k$ molecule. This is because HEL(46-61) binds to $I\text{-}A^k$ better than do other HEL peptides, and does not bind to $I\text{-}E^k$. However, it is not yet known exactly which structural features of a peptide determine immunodominance. As mentioned earlier, for class I–restricted antigen presentation, immunodominant peptides are required to have amino acid residues whose side chains fit into pockets of the MHC molecule–peptide-binding cleft. Common features of immunodominant peptides for class II MHC–restricted antigen presenta-

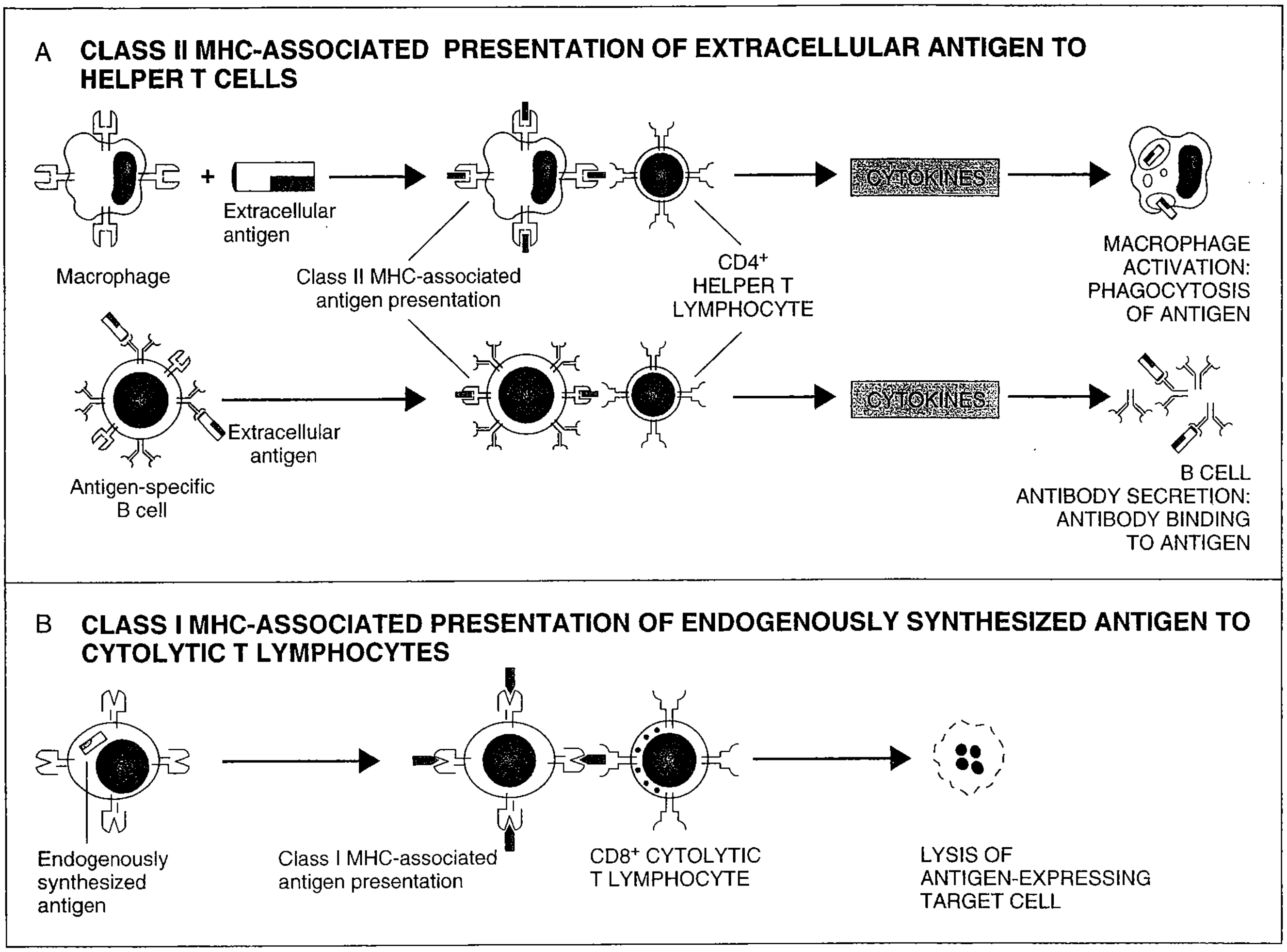


**FIGURE 6-10. Presentation of exogenous and endogenous protein antigens to different subsets of T cells.** MHC, major histocompatibility complex.

tion are less well defined. The question is an important one because an understanding of these features may permit the efficient manipulation of the immune system with synthetic peptides. An obvious application of such knowledge is the design of vaccines. For example, a protein encoded by a viral gene could be analyzed for the presence of amino acid sequences that would form a typical immunodominant secondary structure capable of binding to MHC molecules with high affinity. Vaccines composed of synthetic peptides mimicking this region of the protein theoretically would be effective in eliciting T cell responses against the viral peptide expressed on an infected cell, thereby establishing protective immunity against the virus.

2. *The expression of particular class II MHC alleles in an individual determines the ability of that individual to respond to particular antigens.* The phenomenon of immune response (Ir) gene-controlled immune responsiveness was mentioned in Chapter 5. We now know that Ir genes that control antibody responses are class II MHC genes. They influence immune responsiveness in part because various allelic class II MHC molecules differ in their ability to bind different antigenic peptides and, therefore, to stimulate specific helper T cells. For instance, H-$2^k$ mice are responders to HEL(46-61), but H-$2^d$ mice are non-responders to this epitope. Equilibrium dialysis experiments have shown that HEL(46-61) binds to I-$A^k$ but not to I-$A^d$ molecules. A possible molecular basis for this difference in MHC association is suggested from the model of the class II molecule and the known amino acid sequences of I-$A^k$ and I-$A^d$ proteins. If the HEL(46-61) peptide is hypothetically placed in the predicted binding cleft of the I-$A^k$ molecule, charged residues of the HEL peptide become aligned with oppositely charged residues of the MHC molecule. This would presumably stabilize the bimolecular interaction. In contrast, the I-$A^d$ molecule has different amino acids in the binding cleft that would result in the aligning of like-charged residues with the HEL peptide. Therefore, HEL(46-61) would not bind to or be presented in association with I-$A^d$, and the H-$2^d$ mouse would be a non-responder. Similar results have been obtained with numerous other peptides. MHC-linked immune responsiveness may also be important in humans. For instance, Caucasians who are homozygous for an extended HLA haplotype containing HLA-B8,DR3,

DQw2a are low responders to hepatitis B virus surface antigen. Individuals who are heterozygous at this locus are high responders, presumably because the other alleles contain one or more HLA gene that confers responsiveness to this antigen. Thus, HLA typing may prove to be valuable for predicting the success of vaccination. These findings support the **determinant selection model** of MHC-linked immune responses. This model, which was proposed many years before the demonstration of peptide-MHC binding, states that the products of MHC genes in each individual select which determinants of protein antigens will be immunogenic in that individual. We now understand the structural basis of determinant selection and Ir gene function in antigen presentation. Most Ir gene phenomena have been studied by measuring helper T cell function, but the same principles apply to CTLs. Individuals with certain MHC alleles may be incapable of generating CTLs against some viruses. In this situation, of course, the Ir genes may map to one of the class I MHC loci.

Although these concepts are based largely on studies with simple peptide antigens and inbred strains of mice, they are also relevant to the understanding of immune responses to complex multideterminant protein antigens in outbred species. It is likely that all individuals will express at least one MHC molecule capable of binding at least one determinant of a complex protein, so that all individuals will be responders to such antigens. As stated in Chapter 5, this may be the evolutionary pressure for maintaining MHC polymorphism.

This discussion of the influence of MHC gene products on the immunogenicity of protein antigens has focused on antigen presentation and has not considered the role of the T cells. We have mentioned earlier that the exquisite specificity and diversity of antigen recognition are attributable to antigen receptors on T cells. MHC-linked immune responsiveness is also dependent, in part, on the presence and absence of specific T cells. In fact, some peptides may bind to MHC molecules in a particular inbred mouse strain but do not activate T cells in that strain. It is likely that these mice lack T cells capable of recognizing the particular peptide-MHC complexes. *Thus, Ir genes may function by determining antigen presentation or by shaping the repertoire of antigen-responsive T cells.* The development of the T cell repertoire and the role of the MHC in T cell maturation are discussed in Chapter 8.

## SUMMARY

T cells recognize antigens only on the surface of accessory cells in association with the products of self MHC genes. $CD4^+$ helper T lymphocytes recognize antigens in association with class II MHC gene products (class II MHC–restricted recognition), and $CD8^+$ CTLs recognize antigens in association with class I gene products (class I MHC–restricted recognition). Antigen processing consists of the introduction of protein antigens into APCs, the proteolytic degradation of these proteins into peptides, the binding of peptides to newly assembled MHC molecules, and the display of the peptide-MHC complexes on the APC surface for potential recognition by T cells. Antigen-processing pathways in APCs utilize basic cellular proteolytic mechanisms, which also operate independent of the immune system. Both extracellular and intracellular proteins are sampled by these antigen-processing pathways, and peptides derived from both normal self proteins and foreign proteins are displayed by MHC molecules for surveillance by T lymphocytes. Specialized APCs, including macrophages, B lymphocytes, and dendritic cells, internalize extracellular proteins into endosomes for processing by the class II MHC pathway. These proteins are proteolytically cleaved by enzymes that function at acidic pH in vesicles of the endosomal pathway. Newly synthesized class II MHC heterodimers associate with the invariant chain and are directed from the ER to the endosomal vesicles, where the invariant chain is proteolytically cleaved, and a small peptide remnant of the invariant chain is removed from the peptide binding cleft of the MHC molecule by the DM molecules. The peptides generated from extracellular proteins then bind to the class II MHC molecule, and the trimeric complex (class II MHC $\alpha$ and $\beta$ chains and peptide) moves to the surface of the cell. Cytosolic proteins, usually synthesized in the cells, such as viral proteins, enter the class I MHC pathway of antigen presentation. The proteasome is a cytoplasmic multiprotein complex which proteolytically degrades ubiquitinated cytoplasmic proteins and probably generates a large part of the peptides destined for display by class I MHC molecules. Peptides are delivered from the cytoplasm to the ER by the TAP molecules. Newly formed class I MHC dimers in the ER associate with and bind peptides delivered by TAP. Peptide binding stabilizes class I MHC molecules and permits their movement out of the ER, through the Golgi, to the cell surface. These pathways of MHC-restricted antigen presentation ensure that most of the body's proteins are screened for the possible presence of foreign antigens. The pathways also ensure that proteins from extracellular microbes are likely to generate peptides bound to class II MHC molecules for recognition by $CD4^+$ helper T cells, while proteins encoded by intracellular microbes generate peptides bound to class I MHC molecules for recognition by $CD8^+$ CTLs. The immunogenicity of microbial proteins depends on the ability of antigen-processing pathways to generate peptides from these proteins which bind to self MHC molecules.

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# ATTACHMENT 2

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# ATTACHMENT 3

1: J Immunol. 1979 Oct;123(4):1715-20.



# M467: a murine IgA myeloma protein that binds a bacterial protein. I. Recognition of common antigenic determinants on Salmonella flagellins.

**Smith AM, Miller JS, Whitehead DS.**

We have studied the binding of M467, an IgA murine myeloma protein, to flagellin from seven species of Salmonella. It was found that M467 was reacting with antigenic determinants that were common to all the flagellins studied. These determinants were not related to serotypic antigens. Electronmicrographs of unreduced M467 showed a variety of polymeric species bound to flagella in a manner that could produce immobilization as well as agglutination and precipitation through cross-linking of antigenic determinants. Immunodiffusion in agar gel revealed that M467 was recognizing more than one group of peptide determinants on the flagellins studied. Passive hemagglutination inhibition and a solid phase radioimmunoassay provided evidence that there were differences in binding avidities between M467 and the various Salmonella flagellins studied. It was concluded that M467 is binding more than one specific group of antigenic peptide determinants on flagellin molecules. Flagellin from four of the seven species of Salmonella studied were deficient in one or more of these determinants.

PMID: 90100 [PubMed - indexed for MEDLINE]

# ATTACHMENT 4

1: Arerugi. 1965 Sep;14(9):519-21. Related Articles, Linl

# [Endothelial reaction of peptide antigen of Schistosoma japonicum]

[Article in Japanese]

**Akuzawa M, Tsuchiya S.**

PMID: 5885617 [PubMed - indexed for MEDLINE]

# ATTACHMENT 5

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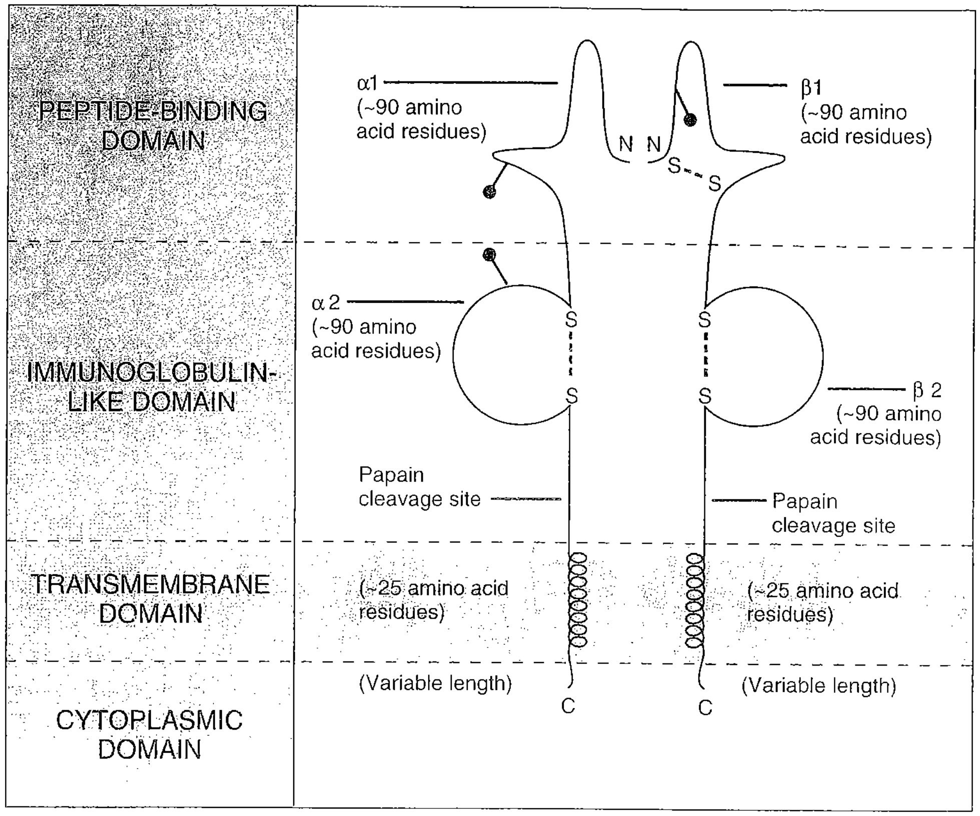


**FIGURE 5-4. Schematic diagram of a class II major histocompatibility complex molecule.** Different segments are not shown to scale. N and C refer to amino and carboxy termini of the polypeptide chains, respectively; S--S, intrachain disulfide bonds; and ⫯ carbohydrate. The papain cleavage sites were used to prepare the extracellular portion of class II molecules for x-ray crystallography.

The class II $\alpha2$ and $\beta2$ segments are essentially nonpolymorphic among various alleles of a particular class II gene but show some differences among the different genetic loci. Thus, the $\alpha2$ regions of all DR alleles are similar, but DR$\alpha2$ differs from DP$\alpha2$ and DQ$\alpha2$. The correlation of CD4 expression on T cells with specificity for class II MHC molecules arises from binding of the CD4 molecule with a projecting loop of the Ig-like nonpolymorphic $\beta2$ domain of the class II molecules, similar to the interaction of CD8 with $\alpha3$ of the class I heavy chain.

The Ig-like regions of the class II molecules are probably important for non-covalent interactions between the two chains, although other portions of the polypeptide chains no doubt contribute as well. These interactions are quite strong and can be disrupted only by harsh denaturing conditions. In general, $\alpha$ chains of one locus (e.g., DR) pair best with $\beta$ chains of the same locus and less commonly with $\beta$ chains of other loci (e.g., DQ or DP).

The carboxy terminal ends of the $\alpha2$ and $\beta2$ segments continue into short connecting regions followed by approximately 25 amino acid stretches of hydrophobic residues that span the membrane. In both chains, the hydrophobic transmembrane region ends with a cluster of basic amino acid residues; these are followed by the carboxy terminal ends of the polypeptides, which form short, hydrophilic cytoplasmic tails.

## The Structural Basis of Peptide Binding to MHC Molecules

Before considering the structural features of the binding of peptides to class I and class II MHC molecules, we will summarize the key features of this interaction that have been deduced from biochemical studies.

1. *The association of antigenic peptides and MHC molecules is a saturable, low-affinity interaction ($K_d \approx 10^{-6}$ M) with a slow "on rate" and a very slow "off rate."* These features were determined first by the techniques of equilibrium dialysis (see Chapter 3, Box 3–3) and gel filtration using purified class II MHC molecules and fluorescently or radioactively labeled peptides. The affinity of peptide-MHC interaction is much lower than that of antigen-antibody binding, which usually has a $K_d$ of $10^{-7}$ to $10^{-11}$ M. In a solution, saturation of peptide binding to class II MHC molecules takes 15 to 30 minutes. Once bound, peptides may stay associated for hours to many weeks! The slow on rate of association of peptides with class II MHC molecules suggests that conformational changes in both peptide and MHC molecule are required before stable binding occurs. Dissociation of peptides from class I molecules is even slower than from class II molecules and usually requires separation of the $\alpha$ chain from $\beta_2$ microglobulin to occur at all. The extraordinarily slow off rates of peptide dissociation from MHC molecules allow peptide-MHC complexes to

persist long enough to interact with T cells despite the low affinity of the interaction.

2. *Each class I or class II MHC molecule binds only one peptide at a time.* This was apparent from the analysis of peptide binding to MHC molecules in solution and was confirmed by the solution of the x-ray crystallographic structure of both class I and class II MHC molecules, which show peptide occupying a single binding cleft.

3. *Multiple different peptides can bind to the same MHC molecule, albeit at different times.* This was first suggested by functional assays in which recognition of one peptide-MHC complex by a T cell could be inhibited by the addition of another structurally similar peptide. In these experiments, the MHC molecule apparently could bind different peptides, but the T cell recognized only one peptide-MHC complex. Definitive evidence for the ability of a single MHC molecule to bind different peptides came from direct binding studies with purified MHC molecules in solution as well as the analyses of peptides eluted from MHC molecules derived from intact cells. Although a wide variety of peptides with diverse amino acid sequences are capable of binding to each MHC molecule, there are certain structural constraints (discussed below) that prohibit all peptides from binding to any individual MHC molecule indiscriminately. These observations, together with the limited number of MHC alleles expressed in each individual, support the hypothesis that *MHC molecules show a broad specificity for peptide binding and that the fine specificity of antigen recognition must reside largely in the antigen receptors of T lymphocytes.*

4. *All of the peptides that bind to a particular allelic form of an MHC molecule show certain common features that may not be shared by peptides that bind to other allelic MHC molecules.* Examples of shared features are a hydrophobic residue at position 2 or a positively charged residue at position 7. Mutagenesis studies have confirmed that such motifs are crucial for peptide binding to particular allelic forms of MHC molecules.

5. *There are distinct differences in the nature of peptides that bind to class I or class II MHC molecules.* Most significantly, peptides that are eluted from class I molecules are typically 9 to 11 amino acid residues in length, whereas those eluted from class II molecules can range from 10 to 30 residues or more.

6. *The amino acid residues that vary among different alleles of class I and class II MHC molecules are largely confined to the amino terminal peptide binding domains.* Mutational analyses of MHC molecules confirm that *many of these polymorphic residues define the peptide binding specificity of the molecule encoded by a particular MHC allele. Other polymorphic residues, also located within the amino terminal peptide binding domains, do not affect peptide binding but do affect T cell recognition of the peptide-MHC molecule complex.*

These features of the peptide-MHC interaction can now be explained in precise structural terms. For example, the $\alpha$-helical sides of the cleft of class I MHC molecules converge at the ends of the cleft, limiting the size of peptides that can be accommodated within the cleft to nine or ten residues. The binding of an 11-residue peptide is possible, but it requires that the peptide bow upward in the center in order to be accommodated. Twelve residues is simply too large to fit into a class I cleft. In contrast, the $\alpha$-helical sides of the cleft of class II MHC molecules do not converge, allowing bound peptides to extend outward from the ends of the cleft. Thus, peptides that bind to class II molecules have no maximum length. This structural difference accounts for the observed difference in the size of peptides eluted from class I versus class II molecules. In class I MHC molecules, the charged amino terminal and carboxy terminal ends of the peptide interact electrostatically with countercharges on the MHC molecule. Such interactions do not occur in class II molecules.

In any given class I or class II MHC molecule, the characteristically conserved features of the peptides that bind to that allelic form of the molecule are complemented by the presence of specific structural features of the MHC molecule such as the presence of pockets in the floor of the cleft. These pockets are actually spaces between the peptide backbones of the $\beta$-pleated strands. The presence or absence of a pocket is determined by the amino acid sequence of the $\beta$ strands, and when a pocket is formed, the polymorphic residues of the MHC molecule that form the pocket determine the nature of the peptide side chain that can fit into the pocket (e.g., hydrophobic, charged, etc.). Conserved peptide residues that fit into the pockets of the MHC molecules are called "anchor residues" because they are critical for attaching the peptide to the MHC molecule. In the initial structures that were solved for class I molecules, the anchor residues were located near the ends of the peptide, placing little constraint upon the peptide sequence except at the ends. Some more recent structures indicate that this feature is not universal (i.e., anchor residues can be located in the middle of the peptide and interact with pockets in the middle of the cleft). Anchor residues make a strong contribution to peptide binding but are not the sole basis of attachment to MHC molecules. Some polymorphic residues in the $\alpha$-helices of the MHC molecule make contacts with the peptide and may also contribute to specificity of binding. Finally, some of the contacts between the MHC molecule and the peptide involve non-polymorphic amino acid residues of the $\alpha$-helices; these residues typically interact with conserved features of the peptide, such as its peptide backbone, and do not contribute to specificity but do stabilize binding.

Some peptide amino acid side chains and

some polymorphic residues of the $\alpha$-helices point upward (i.e., away from the floor of the cleft). These residues do not contribute to peptide-MHC interactions but instead form the antigenic surface recognized by the T cell receptor. In other words, *amino acid side chains from both peptide and MHC molecules contribute to T cell recognition.*

These structural studies have established the significance of polymorphisms within the MHC, namely that *the polymorphic residues of MHC molecules contribute to determining the specificity of peptide binding and to determining the structure recognized by T cell antigen receptors.* We conclude our discussion of peptide binding to MHC molecules with a consideration of the genetic basis of MHC polymorphism. On a population level, there is an advantage to having multiple alleles, namely that the presence of polymorphism decreases the likelihood that any particular microbe can escape detection by the immune systems of all individuals in the population by encoding proteins that cannot be digested into peptides capable of binding to some host MHC molecule. It is hard to calculate how significant an advantage to the population MHC polymorphism actually confers because, as we have described, the structural requirements for peptide binding by particular MHC alleles are fairly broad. Furthermore, many different allelic forms of MHC molecules may have very similar binding specificities for peptides, an observation that has led some investigators to divide MHC molecules into a limited number of "supertypes." Despite the obvious advantage to the population of having widely polymorphic MHC molecules, it has not been possible to demonstrate that infectious microbes have exerted selective pressure on generating or maintaining specific polymorphisms. Nevertheless, specific mechanisms have evolved for generating new MHC molecule polymorphisms, which we will discuss when we consider the genomic organization of the MHC.

## GENOMIC ORGANIZATION OF THE MHC

### Organization of the MHC Gene Loci

In humans, the MHC is located on the short arm of chromosome 6. $\beta_2$ microglobulin is encoded by a gene on chromosome 15. The human MHC occupies a large segment of DNA, extending about 3500 kilobases (kb). (For comparison, a large human gene may extend up to 50 to 100 kb, and 3500 kb is the size of the entire *Escherichia coli* genome!) In classical genetic terms, it extends about 4 centimorgans, meaning crossovers within the MHC occur with a frequency of over 4 per cent at each meiosis. A recent molecular map of the human MHC is shown in Figure 5–5. Many of the genes found within the MHC code for proteins whose function is not yet known. In addition, there are many as yet unidentified genes, especially within the class I region. Remarkably, expression of almost all of the genes located within the MHC is responsive to the cytokine, interferon-$\gamma$ (IFN-$\gamma$). The class II genes are located closest to the centromere.

A surprise from these gene-mapping studies is that there may be two or three functional $\beta$ chain genes for some class II loci but usually only one

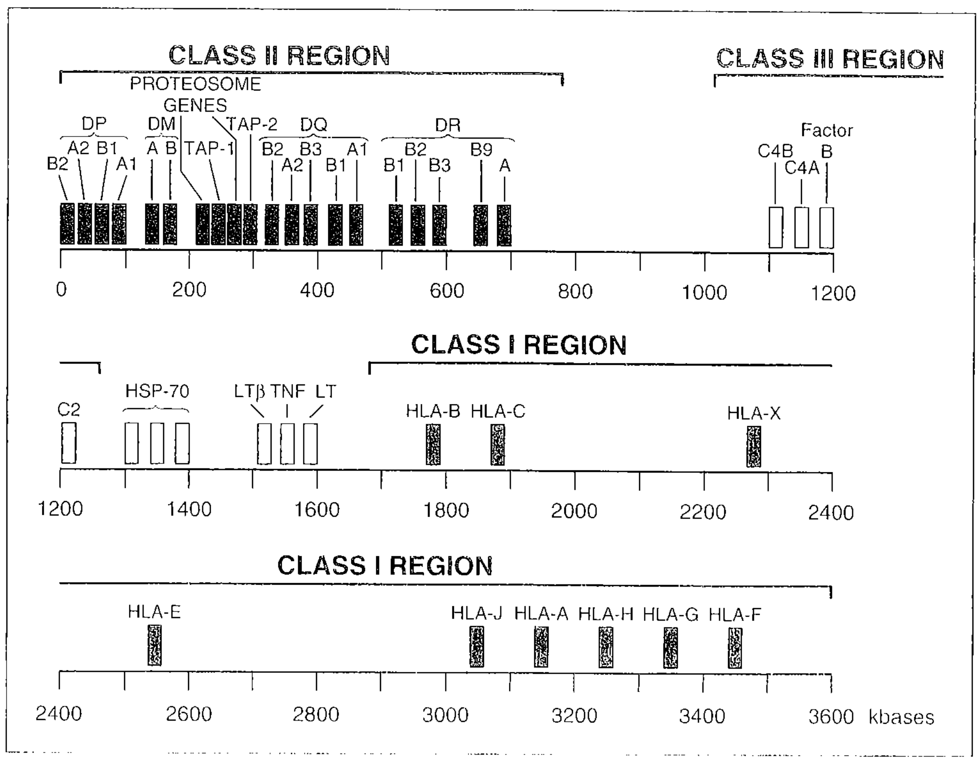


**FIGURE 5–5. Molecular map of the human major histocompatibility complex.** HLA-F, G, H, J, and X are class I–like molecules. This map is simplified to exclude other class I–and class II–like genes, genes not of immunologic interest, and numerous genes of unknown function. The pattern of class II genes may vary with the inherited allele. DM, TAP, and proteosome genes contribute to MHC molecule assembly; C2, C4A, C4B, and Factor B are complement proteins; HSP-70 is a heat shock protein; lymphotoxin (LT), lymphotoxin $\beta$ (LT-$\beta$), and tumor necrosis factor (TNF) are cytokine genes.

mined for many different peptides by assessing the effects of single amino acid substitutions on both T cell recognition and MHC binding (see Table 7–2).

The affinity of TCR binding to peptide-MHC complexes is believed to be significantly lower, on average, than Ig binding to antigen. This may be, in part, due to selection processes in the thymus that favor development of T cells with low affinity TCRs (see Chapter 8). Crystallographic data also suggest that the TCR is a much more rigid structure than Ig, with less flexibility at the hinge regions or between C and V domains. This is in keeping with the restricted TCR recognition of only peptide-MHC complexes and not other structures.

Whether a particular T cell is class I or class II MHC–restricted is generally not determined by the V, D, J, or C gene sequences in the $\alpha$ or $\beta$ chain of the TCR of that cell, although there may be some predilection for the selective use of certain V genes in $CD4^+$ versus $CD8^+$ T cells. Overall, the same sets of TCR genes can be expressed in class I– and class II–restricted T cells. As we mentioned in Chapter 6 and will discuss in more detail later in this chapter, the ability of a particular T cell to respond to either class I– or class II–associated peptide is determined mainly by the expression of CD8 or CD4, respectively.

## THE CD3, $\zeta$, AND $\eta$ PROTEINS ASSOCIATED WITH THE TCR COMPLEX

The $\alpha\beta$ TCR heterodimer provides T cells the ability to recognize peptide antigens bound to MHC molecules, but *both the cell surface expression of TCR molecules and their function in activating T cells are dependent on up to five other transmembrane proteins that non-covalently associate with the $\alpha\beta$ heterodimer.* Together, these proteins form the functional **TCR complex** (Fig. 7–5 and Table 7–1). Three proteins in the complex are called **CD3 molecules,** and include highly homologous Ig superfamily members designated $\gamma$, $\delta$, and $\epsilon$. In addition, 80 to 90 per cent of TCR complexes contain a disulfide-linked homodimer of a protein called the **$\zeta$ chain** that does not belong to the Ig gene superfamily. The remaining 10 per cent of human T cells express heterodimers consisting of the $\zeta$ chain and the highly homologous Fc$\epsilon$RI $\gamma$ chain (see Chapter 14). In mice, 10 to 20 per cent of T cells express a heterodimer of the $\zeta$ chain and an alternative splice product of the $\zeta$ gene called the $\eta$ chain. The exact stoichiometry of TCR complexes is not known but models have been proposed based on the molecular weights and molar ratios of immunoprecipitated TCR components, as well as consideration of charged residues in transmembrane segments of the TCR $\alpha$ and $\beta$ chains and the CD3 chains. A likely stoichiometry of the most common form of TCR is $(\alpha\beta)_2\epsilon_2\gamma\delta\zeta\text{-}\zeta$.

### Structure and Association of CD3, $\zeta$, and $\eta$ Proteins

The CD3 proteins were first identified, before the $\alpha\beta$ heterodimer, by the use of monoclonal antibodies raised against T cells, and the $\zeta$ and $\eta$ chains were identified later by co-immunoprecipitation with $\alpha\beta$ and CD3 proteins. The physical association of the $\alpha\beta$ heterodimer, CD3, and $\zeta$ chains has been demonstrated in two ways.

1. Antibodies against the $\alpha\beta$ TCR heterodimer or the CD3 proteins co-precipitate both the heterodimer and the associated proteins from solubilized cell membrane preparations.
2. When intact T cells are treated with either anti-CD3 or anti-$\alpha\beta$ heterodimer antibodies, the en-

**TABLE 7–2.** Identification of MHC-Binding and T Cell Receptor–Binding Residues in Peptide Antigens

| | HEL Peptide | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *Amino Acid Residue Position No.* | | | | | | | | | | *Stimulation of HEL-Specific T Cells* | *Binding to Purified I-A$^d$* | *Competition with Native HEL for T Cell Stimulation* |
| | 52 | 53 | 54 | 55 | 56 | 57 | 58 | 59 | 60 | 61 | | | |
| 1 | Asp | Tyr | Gly | Ile | Leu | Gln | Ile | Asn | Ser | Arg | + | + | NA |
| 2 | Asp | Tyr | Gly | Ile | *Ala* | Gln | Ile | Asn | Ser | Arg | – | + | + |
| 3 | Asp | *Ala* | Gly | Ile | Leu | Gln | Ile | Asn | Ser | Arg | – | + | + |
| 4 | Asp | Tyr | Gly | *Ala* | Leu | Gln | Ile | Asn | Ser | Arg | – | – | – |
| 5 | Asp | Tyr | *Ala* | Ile | Leu | Gln | Ile | Asn | Ser | Arg | + | + | NA |

Synthetic peptides were produced that differed from the native hen egg lysozyme peptide HEL (52-61) (peptide 1) by substitutions for single residues, and the functional consequences of these engineered mutations were analyzed. Substitutions at positions 56 and 53 (peptides 2 and 3) result in loss of T cell stimulation, but retain I-A binding. The amino acids at these positions in the native peptide are part of the epitope recognized by the T cell receptor. Substitution of residue 55 (peptide 4) results in loss of T cell stimulation and I-A binding. This residue is in part of the peptide that binds to the class II MHC molecule. A substitution at position 54 (peptide 5) has no effect, and therefore this residue is not essential for binding of the peptide to either the MHC or T cell receptor molecules.

*Abbreviations:* MHC, major histocompatibility complex; HEL, hen egg lysozyme; NA, not applicable.

Adapted with permission from Unanue, E. R., and P. M. Allen. The basis for the immunoregulatory role of macrophages and other accessory cells. Science 236:551–557, 1987. Copyright 1987 American Association for the Advancement of Science.

# ATTACHMENT 6

SAUNDERS TEXT AND REVIEW SERIES

# CELLULAR AND MOLECULAR IMMUNOLOGY

THIRD EDITION

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for immunization. The screening method depends on the antigen being used. For soluble antigens, the usual technique is RIA or ELISA, and for cell surface antigens, a variety of assays for antibody binding to viable cells can be used (see Laboratory Uses of Antibodies later in this chapter). Once positive wells are identified (i.e., wells containing hybridomas producing the desired antibody), the cells are cloned in semisolid agar or by limiting dilution, and clones producing the antibody are isolated by another round of screening. These cloned hybridomas produce monoclonal antibodies of a desired specificity. Hybridomas can be grown in large volumes or as ascitic tumors in syngeneic mice in order to produce large quantities of monoclonal antibodies.

Two features of this somatic cell hybridization make it extremely valuable. First, it is the best method for producing a monoclonal antibody against a known antigenic determinant. Second, it can be used to identify unknown antigens present in a mixture because each hybridoma is specific for only one antigenic determinant. For instance, if several hybridomas are produced that secrete antibodies that bind to the surface of a particular cell, each hybridoma clone will secrete an antibody specific for only one surface antigenic determinant. These monoclonal antibodies can then be used to purify different cell surface molecules, some of which may be known molecules and others that may not have been identified previously. Some of the commonest applications of hybridomas and monoclonal antibodies include the following:

(1) Identification of phenotypic markers unique to particular cell types. The basis for the modern classification of lymphocytes and mononuclear phagocytes is the binding of population-specific monoclonal antibodies. These have been used to define "clusters of differentiation" for various cell types (see Chapter 2).
(2) Immunodiagnosis. The diagnosis of many infectious and systemic diseases relies upon the detection of specific antigens and/or antibodies in the circulation or in tissues, using monoclonal antibodies in immunoassays.
(3) Tumor diagnosis and therapy. Tumor-specific monoclonal antibodies are used for detection of tumors by imaging techniques and for immunotherapy of tumors *in vivo*.
(4) Functional analysis of cell surface and secreted molecules. In immunologic research, monoclonal antibodies that bind to cell surface molecules and either stimulate or inhibit particular cellular functions are invaluable tools for defining the functions of surface molecules, including receptors for antigens. Antibodies that neutralize cytokines are routinely used for detecting the presence and functional roles of these protein hormones *in vitro* and *in vivo*.

At present, hybridomas are most often produced by fusing HAT-sensitive mouse myelomas with B cells from immunized mice, rats, or hamsters. The same principle is used to generate mouse T cell hybridomas, by fusing T cells with a HAT-sensitive, T cell–derived tumor line; uses of such monoclonal T cell populations are described in Chapter 7. Attempts are being made to generate human monoclonal antibodies, primarily for administration to patients, by developing human myeloma lines as fusion partners. (It is a general rule that the stability of hybrids is low if cells from species that are far apart in evolution are fused, and this is presumably why human B cells do not form hybridomas with mouse myeloma lines at high efficiency.) As we shall discuss later in the chapter, only small portions of the antibody molecule are responsible for binding to antigen; the remainder of the antibody molecule can be thought of as a "framework." This structural organization allows the DNA segments encoding the antigen-binding sites from a murine monoclonal antibody to be "stitched" into a complementary DNA encoding a human myeloma protein, creating a hybrid gene. When expressed, the resultant hybrid protein, which retains antigen specificity, is referred to as a "humanized antibody." Humanized antibodies offer an alternative strategy for generating monoclonal antibodies that may be safely administered to patients.

When blood or plasma forms a clot, antibodies remain in the residual fluid, called **serum.** A sample of serum that contains a large number of antibody molecules that bind to a particular antigen is commonly called an **antiserum.** (The study of antibodies and their reactions with antigens is therefore classically called **serology.**) The number of antibody molecules in a serum specific for a particular antigen is often measured by serially diluting the serum until binding can no longer be observed; sera with a large number of antibody molecules specific for a particular antigen are said to be "strong" or have a "high titer."

Plasma or serum glycoproteins are traditionally separated by solubility characteristics into albumins and globulins and may be further separated by migration in an electric field, a process called electrophoresis. Elvin Kabat and colleagues demonstrated that most antibodies are found in the third fastest migrating group of globulins, named **gamma globulins** for the third letter of the Greek alphabet. Another common name for antibody is **immunoglobulin** (Ig), referring to the immunity-conferring portion of the gamma globulin fraction. The terms immunoglobulin and antibody are used interchangeably throughout this book.

Currently, antibody molecules are generally purified from plasma or other natural fluids by a two-step procedure. The first step is to precipitate antibodies from the biologic fluid by adding a concentration of ammonium sulfate that ranges from 40 to 50 per cent of saturation. Under these conditions, albumin and most small molecules remain in solution, so that partially purified antibody can be collected in a pellet by centrifugation. The antibody-containing pellet is redissolved in buffer and then purified by various forms of chromatography (the second step). When the antibody of interest in the biologic fluid is specific for a known antigen, the antigen can be immobilized on a column matrix and used to bind the antibody, a method called *affinity chromatography.* Antibody can be recovered from the column matrix by a change in pH.

## Overview of Antibody Structure

A number of the structural and functional features of antibodies were determined from the early studies of these molecules:

1. *All antibody molecules are similar in overall structure, accounting for certain common physico-*

*chemical features, such as charge and solubility.* These common properties may be exploited as a basis for the purification of antibody molecules from fluids such as blood. *All antibodies have a common core structure of two identical light chains (each about 24 kilodaltons [kD]) and two identical heavy chains (about 55 or 70 kD)* (Fig. 3–1). One light chain is attached to each heavy chain, and the two heavy chains are attached to each other. Both the light chains and the heavy chains contain a series of repeating, homologous units, each about 110 amino acid residues in length, which fold independently in a common globular motif, called an **immunoglobulin domain** (Fig. 3–2). All Ig domains contain two layers of $\beta$-pleated sheet with three or four strands of antiparallel polypeptide chain. Certain Ig domains, such as those comprising variable regions (see later), have an extra strand in each of the two layers. As will be discussed in Chapter 7, many other proteins of importance in the immune system contain regions that use the same folding motif and show structural relatedness to Ig amino acid sequences. All molecules that contain this motif are said to belong to the **Ig superfamily,** and all of the gene segments encoding the Ig-like domains are believed to have evolved from the same common ancestral gene (see Chapter 7, Box 7–2).

2. Despite their overall similarity, *antibody molecules can be readily divided into a small number of distinct classes and subclasses, based on minor differences in physicochemical characteristics such as size, charge, and solubility and on their behavior as antigens* (Box 3–2). The classes of antibody molecules are also called **isotypes** and in humans are named IgA, IgD, IgE, IgG, and IgM (Table 3–1). IgA and IgG isotypes can be further subdivided into closely related subclasses, or subtypes, called IgA1 and IgA2, and IgG1, IgG2, IgG3, and IgG4, respectively. In certain instances, it will be convenient to refer to studies of mouse antibody. Mice have the same general isotypes as humans, but the IgG isotype is divided into the IgG1, IgG2a, IgG2b, and IgG3 subclasses. The heavy chains of all antibody molecules of an isotype or subtype share extensive regions of amino acid sequence identity but differ from antibodies belonging to other isotypes or subtypes. Heavy chains are designated by the letter of the Greek alphabet corresponding to the overall isotype of the antibody: IgA1 contains $\alpha 1$ heavy chains; IgA2, $\alpha 2$; IgD, $\delta$; IgE, $\epsilon$; IgG1, $\gamma 1$; IgG2, $\gamma 2$; IgG3, $\gamma 3$, IgG4, $\gamma 4$; and

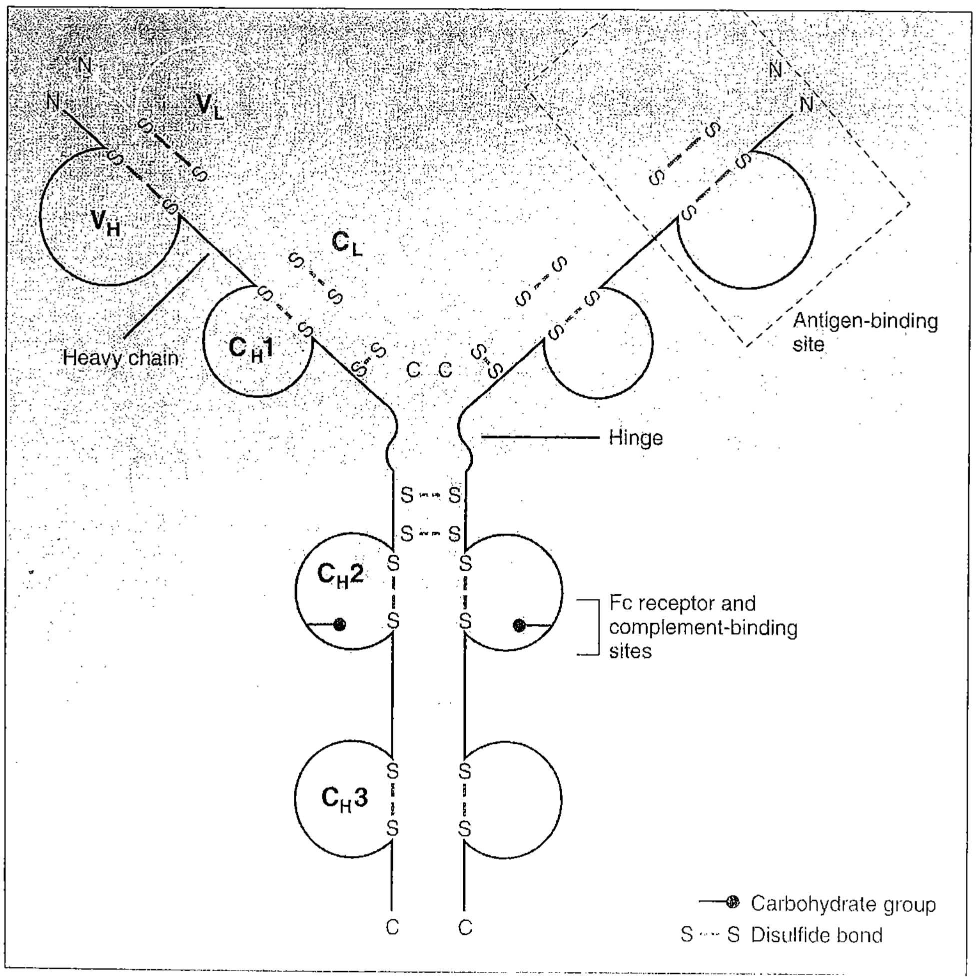


**FIGURE 3–1. Schematic diagram of an immunoglobulin (Ig) molecule.** In this drawing of an IgG molecule, the antigen-binding sites are formed by the juxtaposition of $V_L$ and $V_H$ domains. The locations of complement and Fc receptor-binding sites within the heavy chain constant regions are approximations. S--S refers to intrachain and interchain disulfide bonds; N and C refer to amino and carboxy termini of the polypeptide chains, respectively.

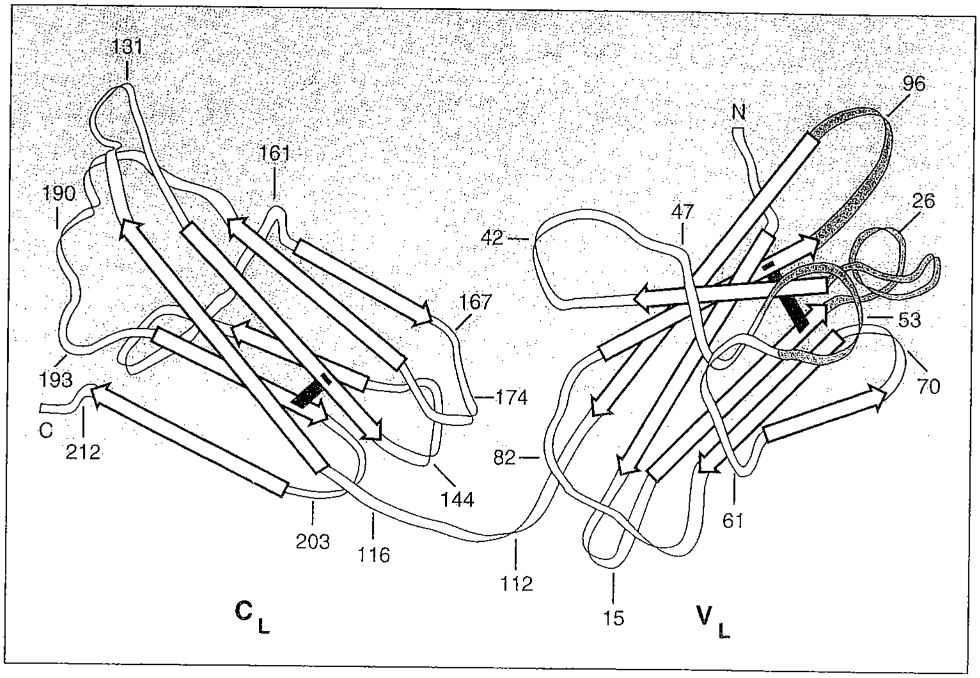


**FIGURE 3–2. Polypeptide folding into immunoglobulin (Ig) domains in a human antibody light chain.** The V and C regions each independently fold into Ig domains. The white arrows represent polypeptide arranged in $\beta$-pleated sheets, the dark blue bars are intrachain disulfide bonds, and the numbers indicate the positions of amino acid residues counting from the amino (N) terminus. The CDR1, CDR2, and CDR3 loops of the V region, colored in light blue, are brought together to form the antigen-binding surface of the light chain. (Adapted with permission from Edmundson, A. B., K. R. Ely, E. E. Abola, M. Schiffer, and N. Panagiotopoulos. Rotational allostery and divergent evolution of domains in immunoglobulin light chains. Biochemistry 14:3953–3961, 1975. Copyright 1975, American Chemical Society.)

IgM, $\mu$. The shared regions of heavy chain amino acid sequences are responsible for both the common physicochemical properties and the common antigenic properties of antibodies of the same isotype. In addition, the shared regions of the heavy chains provide members of each isotype with common abilities to bind to certain cell surface receptors or to other macromolecules like complement and thereby activate particular immune effector functions. Thus, the separation of antibody molecules into isotypes and subtypes on the basis of common structural features also separates antibodies according to which set of effector functions they commonly activate. In other words, *different effector functions of antibodies are mediated by distinct isotypes and subtypes*. As we shall see later, there are two isotypes of antibody light chains, called $\kappa$ and $\lambda$. The light chains do not mediate or influence the effector functions of antibodies. However, as we shall discuss shortly, both the heavy and light chains contribute to specific antigen recognition.

3. *There are more than $1 \times 10^7$, and perhaps as many as $10^9$, structurally different antibody molecules in every individual, each with unique amino acid*

**TABLE 3–1.** Human Antibody Isotypes*

| Antibody | Subtypes | H Chain (Designation) | H Chain Domains (Number) | Hinge | Tail Piece | Serum Concentration (mg/ml) | Secretory Form | Molecular Size of Secretory Form (kD) |
|---|---|---|---|---|---|---|---|---|
| IgA | IgA1 | $\alpha$1 | 4 | Yes | Yes | 3 | Monomer, dimer, trimer | 150, 300, or 400 |
| | IgA2 | $\alpha$2 | 4 | Yes | Yes | 0.5 | Monomer, dimer, trimer | 150, 300, or 400 |
| IgD | None | $\delta$ | 4 | Yes | Yes | Trace | — | 180 |
| IgE | None | $\epsilon$ | 5 | No | No | Trace | Monomer | 190 |
| IgG | IgG1 | $\gamma$1 | 4 | Yes | No | 9 | Monomer | 150 |
| | IgG2 | $\gamma$2 | 4 | Yes | No | 3 | Monomer | 150 |
| | IgG3 | $\gamma$3 | 4 | Yes | No | 1 | Monomer | 150 |
| | IgG4 | $\gamma$4 | 4 | Yes | No | 0.5 | Monomer | 150 |
| IgM | None | $\mu$ | 5 | No | Yes | 1.5 | Pentamer | 950 |

* Multimeric forms of IgA and IgM are associated with J chain via the tail piece region of the heavy chain. IgA in mucus is also associated with secretory piece.
*Abbreviations:* Ig, immunoglobulin; kD, kilodalton.

# ATTACHMENT 7

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# United States Patent [19]

Bonneville

[11] Patent Number: 5,723,309

[45] Date of Patent: Mar. 3, 1998

[54] **PRODUCTION OF SUBUNITS OF SOLUBLE T CELL RECEPTORS BY CO-TRANSFECTION**

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[51] **Int. Cl.**[6] .......................... **C12N 15/12**; C12N 15/63; C12N 15/79; C12N 15/87

[52] **U.S. Cl.** ............................. **435/69.1**; 435/4; 435/7.1; 435/69.52; 435/69.7; 435/172.3

[58] **Field of Search** ............................... 435/4, 7.1, 69.1, 435/69.52, 69.7, 172.3; 424/85.2; 514/8

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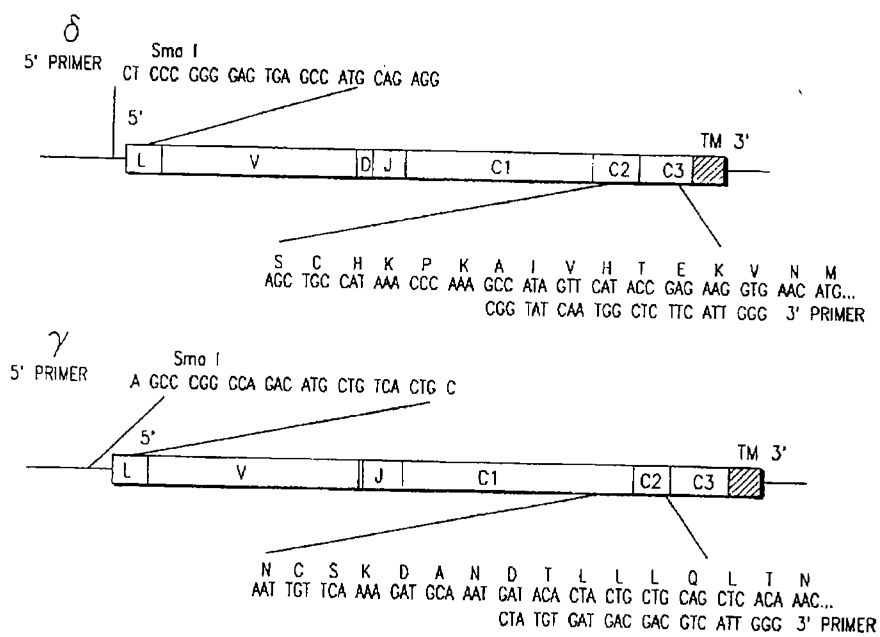
*Assistant Examiner*—Elizabeth C. Kemmerer
*Attorney, Agent, or Firm*—Young & Thompson

[57] **ABSTRACT**

Soluble, single chain T cell receptors, nucleic acid sequences, particularly DNA sequences, encoding the soluble, single chain T cell receptor, expression vectors containing the DNA sequences, and host cells containing the expression vectors.

**10 Claims, 6 Drawing Sheets**



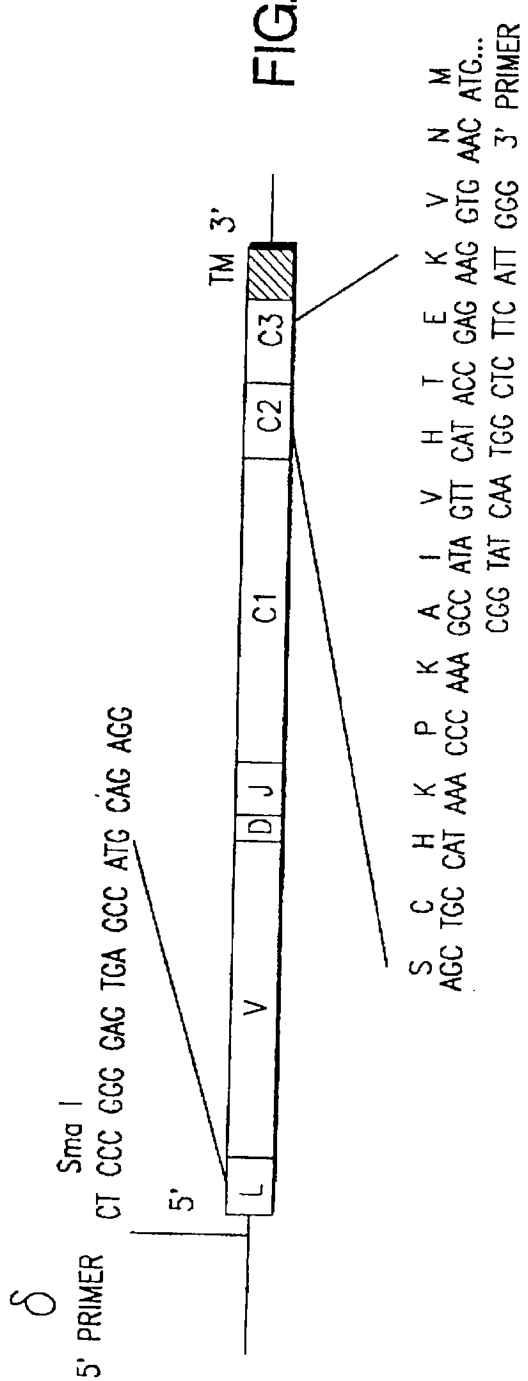

δ
5' PRIMER
5'
Sma I
CT CCC GGG GAG TGA GCC ATG CAG AGG
L
V
D
J
C1
C2
C3
TM 3'
S C H K P K A I V H T E K V N M
AGC TGC CAT AAA CCC AAA GCC ATA GTT CAT ACC GAG AAG GTG AAC ATG...
CGG TAT CAA TGG CTC TTC ATT GGG 3' PRIMER

FIG. 1A

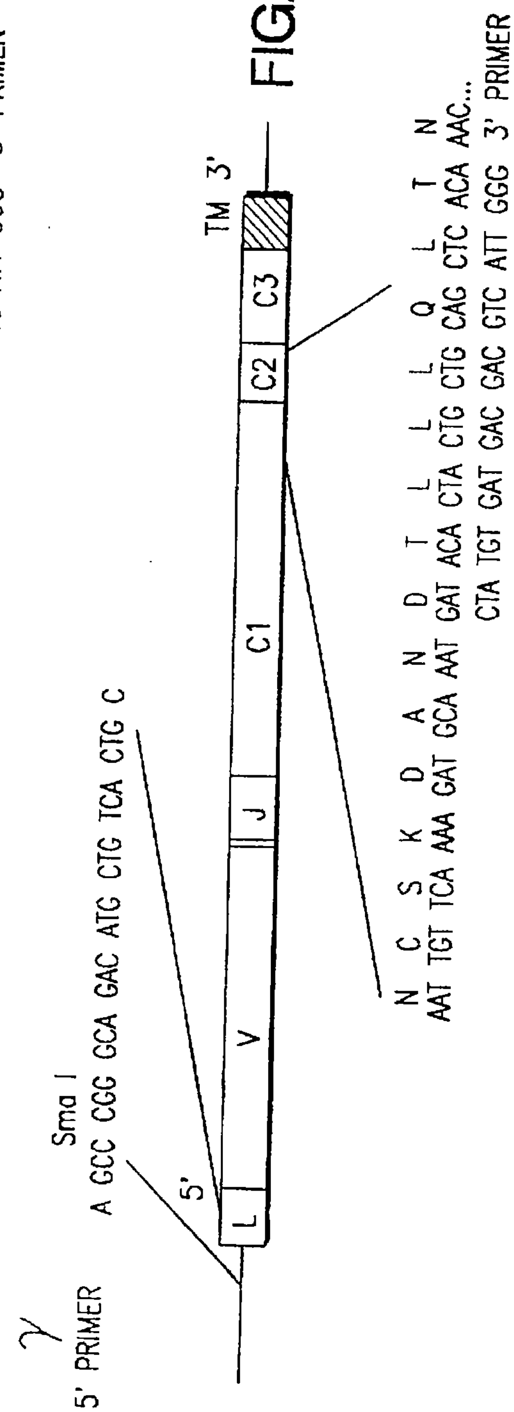

γ
5' PRIMER
5'
Sma I
A GCC CGG GCA GAC ATG CTG TCA CTG C
L
V
J
C1
C2
C3
TM 3'
N C S K D A N D T L L L L Q L T N
AAT TGT TCA AAA GAT GCA AAT GAT ACA CTA CTG CTG CAG CTC ACA AAC...
CTA TGT GAT GAC GAC GTC ATT GGG 3' PRIMER

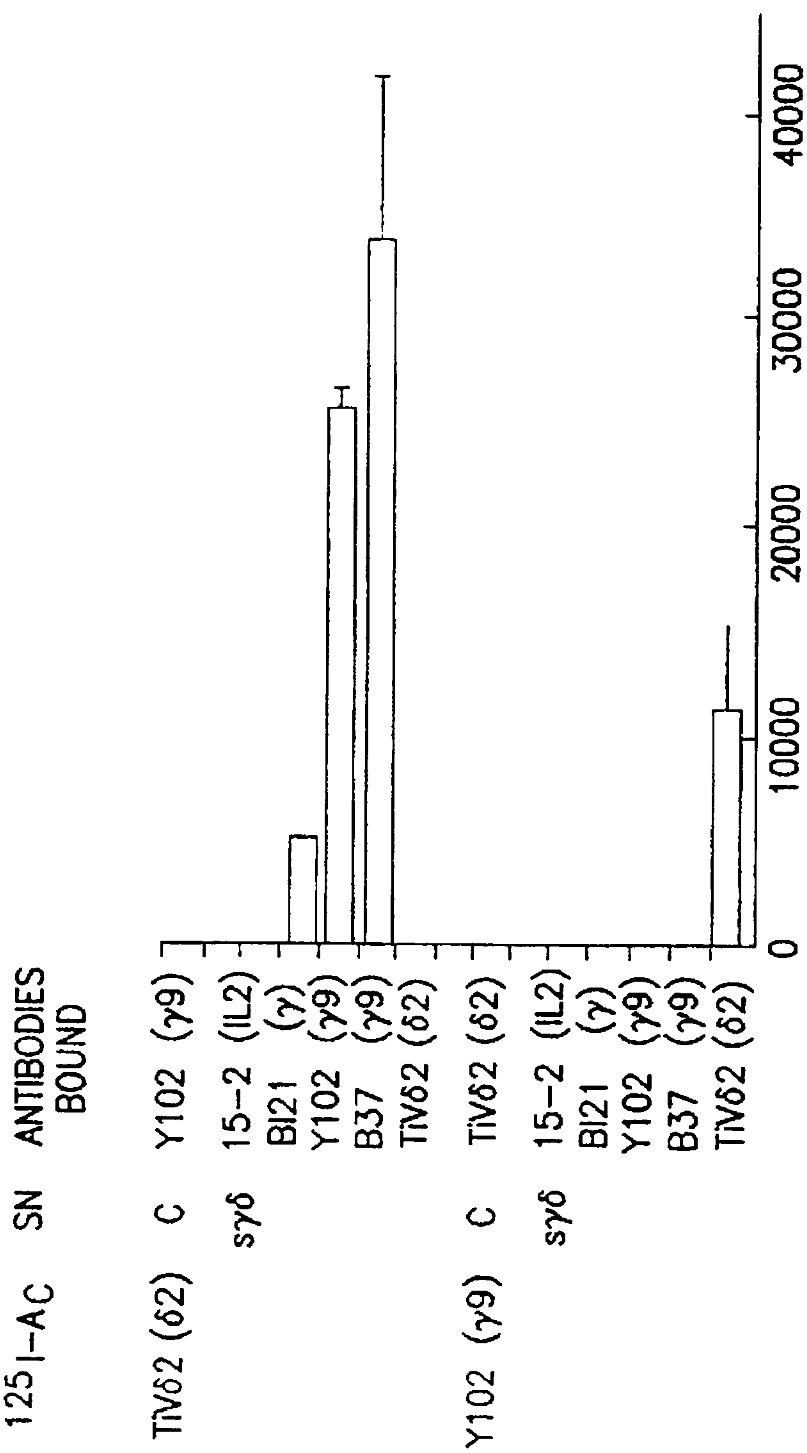
FIG. 1B

16 / 1 46 / 11
ATG CAG AGG ATC TCC TCC CTC ATC CAT CTC TCT CTC TTC TGG GCA GGA GTC ATG TCA GCC
met gln arg ile ser ser leu ile his leu ser leu phe trp ala glv val met ser ala
76 / 21 106 / 31
ATT GAC TTG GTG CCT GAA CAC CAA ACA GTG CCT GTG TCA ATA GGG GTC CCT GCC ACC CTC
ile glu leu val pro glu his gln thr val pro val ser ile gly val pro ala thr leu
136 / 41 166 / 51
AGG TGC TCC ATG AAA GGA GAA GCG ATC GGT AAC TAC TAT ATC AAC TGG TAC AGG AAG ACC
arg cys ser met lys gly glu ala ile gly asn tyr tyr ile asn trp tyr arg lys tnr
196 / 61 226 / 71
CAA GCT AAC ACA ATG ACT TTC ATA TAC CGA GAA AAG GAC ATC TAT GGC CCT CCT TTC AAA
gln gly asn thr met thr phe ile tyr arg glu lys asp ile tyr gly pro gly pne lys
256 / 81 286 / 91
GAC AAT TTC CAA GGT GAC ATT GAT ATT GCA AAG AAC CTG GCT GTA CTT AAG ATA CTT GCA
asp asn phe gln gly asp ile asp ile ala lys asn leu ala val leu lvs ile leu ala
316 / 101 346 / 111
CCA TCA GAG AGA GAT GAA GGG TCT TAC TAC TGT GCC TGT GAC ACC TTG GGG ATG GGG GGG
pro ser glu arg asp glu gly ser tyr tyr cys ala cys asp thr leu gly met gly gly
376 / 121 406 / 131
GAA TAC ACC GAT AAA CTC ATC TTT GGA AAA GGA ACC CGT GTG ACT GTG GAA CCA AGA AGT
glu tyr thr asp lys leu ile pne gly lys gly thr arq val thr val glu pro arg ser
436 / 141 466 / 151
CAG CCT CAT ACC AAA CCA TCC GTT TTT GTC ATG AAA AAT GGA ACA AAT GTC GCT TGT CTG
gin pro nis tnr lys pro ser val pne val met lys asn gly thr asn vla ala cys leu
496 / 161 526 / 171
GTG AAG GAA TTC TAC CCC AAG GAT ATA AGA ATA AAT CTC GTG TCA TCC AAS AAG ATA ACA
val lys glu phe tyr pro lys asp ile arg ile asn leu val ser ser lys lys ile tnr
556 / 181 586 / 191
GAG TTT GAT CCT GCT ATT GTC ATC TCT CCC AGT GGG AAG TAC AAT GCT GTC AAG CTT GGT
glu pne asp pro ala ile val ile ser pro ser gly lys tyr asn ala val lys leu gly
616 / 201 646 / 211
AAA TAT GAA GAT TCA AAT TCA GTG ACA TGT TCA GTT CAA CAC GAC AAT AAA ACT GTG CAC
lys tyr glu asp ser asn ser val thr cys ser val gln his asp asn lys thr val his
676 / 221 706 / 231
TCC ACT GAC TTT GAA GTG AAG ACA GAT TCT ACA GAT CAC GTA AAA CCA AAG GAA ACT GAA
ser thr asp phe glu val lys thr asp ser thr asp his val lys pro lys glu thr glu
736 / 241 766 / 251
AAC ACA AAG CAA CCT TCA AAG AGC TGC CAT AAA CCC AAA GCC ATA GTT CAT ACC GAG AAG
asn thr lys gln pro ser lys ser cys his lys pro lys ala ile val his thr glu lys
796 / 261
TAA
OCH

FIG. 2A

12 / 1 42 / 11
ATG CTG TCA CTG CTC CAC GCA TCA ACG CTG GCA GTC CTT GGG GCT CTG TGT GTA TAT GGT
met leu ser leu leu his ala ser thr leu ala val leu gly ala leu cys val tyr gly
72 / 21 102 / 31
GCA GGT CAC CTA GAG CAA CCT CAA ATT TCC AGT ACT AAA ACG CTG TCA AAA ACA GCC CGC
ala gly his leu glu gln pro gln ile ser ser thr lys thr leu ser lys thr ala arg
132 / 41 162 / 51
CTG GAA TGT GTG GTG TCT GGA ATA ACA ATT TCT GCA ACA TCT GTA TAT TGG TAT CGA GAG
leu glu cys val val ser gly ile thr ile ser ala thr ser val tyr trp tyr arg glu
192 / 61 222 / 71
AGA CCT GGT GAA GTC ATA CAG TTC CTG GTG TCC ATT TCA TAT GAC GGG ACT GTC AGA AAG
arg pro gly glu val ile gln phe leu val ser ile ser tyr asp gly thr val arg lys
252 / 81 282 / 91
GAA TCC GGC ATT CCG TCA GGC AAA TTT GAG GTG GAT AGG ATA CCT GAA ACG TCT ACA TCC
glu ser gly ile pro ser gly lys phe glu val asp arg ile pro glu thr ser thr ser
312 / 101 342 / 111
ACT CTC ACC ATT CAC AAT GTA GAG AAA CAG GAC ATA GCT ACC TAC TAC TGT GCC TTG TGG
thr leu thr ile his asn val glu lys gln asp ile ala thr tyr tyr cys ala leu trp
372 / 121 402 / 131
GAG GCC CAG CAA GAG TTG GGC AAA AAA ATC AAG GTA TTT GGT CCC GGA ACA AAG CTT ATC
glu ala gln gln glu leu gly lys lys ile lys val phe gly pro gly thr lys leu ile
432 / 141 462 / 151
ATT ACA GAT AAA CAA CTT GAT GCA GAT GTT TCC CCC AAG CCC ACT ATT TTT CTT CTT TCA
ile thr asp lys gln leu asp ala asp val ser pro lys pro thr ile phe leu pro ser
492 / 161 522 / 171
ATT GCT GAA ACA AAG CTC CAG AAG GCT GGA ACA TAC CTT TGT CTT CTT GAG AAA TTT TTC
ile ala glu thr lys leu gln lys ala gly thr tyr leu cys leu leu glu lys phe phe
552 / 181 582 / 191
CCT GAT GTT ATT AAG ATA CAT TGG GAA GAA AAG AAG AGC AAC ACG ATT CTG GGA TCC CAG
pro asp val ile lys ile his trp glu glu lys lys ser ash thr ile leu gly ser gln
612 / 201 642 / 211
GAG GGG AAC ACC ATG AAG ACT AAT GAC ACA TAC ATG AAA TTT AGC TGG TTA ACG GTG CCA
glu gly asn thr met lys thr asn asp thr tyr met lys phe set trp leu thr val pro
672 / 221 702 / 231
GAA AAG TCA CTG GAC AAA GAA CAC AGA TGT ATC GTC AGA CAT GAG AAT AAT AAA AAC GCA
glu lys ser leu asp lys glu his arg cys ile val arg his glu asn asn lys asn gly
732 / 241 762 / 251
GTT GAT CAA GAA ATT ATC TTT CCT CCA ATA AAG ACA GAT GTC ATC ACA ATG GAT CCC AAA
val asp gln glu ile ile phe pro pro ile lys thr asp val ile thr met asp pro lys
792 / 261 822 / 271
GAC AAT TGT TCA AAA GAT GCA AAT GAT ACA CTA CTG CTG CAG TAA
asp asn cys ser lys asp ala asn asp thr leu leu leu gln OCH

FIG. 2B



125I-AC
SN
ANTIBODIES BOUND
TiVδ2 (δ2)
C
Y102 (γ9)
sγδ
15–2 (IL2)
BI21 (γ)
Y102 (γ9)
B37 (γ9)
TiVδ2 (δ2)
Y102 (γ9)
C
TiVδ2 (δ2)
sγδ
15–2 (IL2)
BI21 (γ)
Y102 (γ9)
B37 (γ9)
TiVδ2 (δ2)
0
10000
20000
30000
40000
cpm


FIG. 3

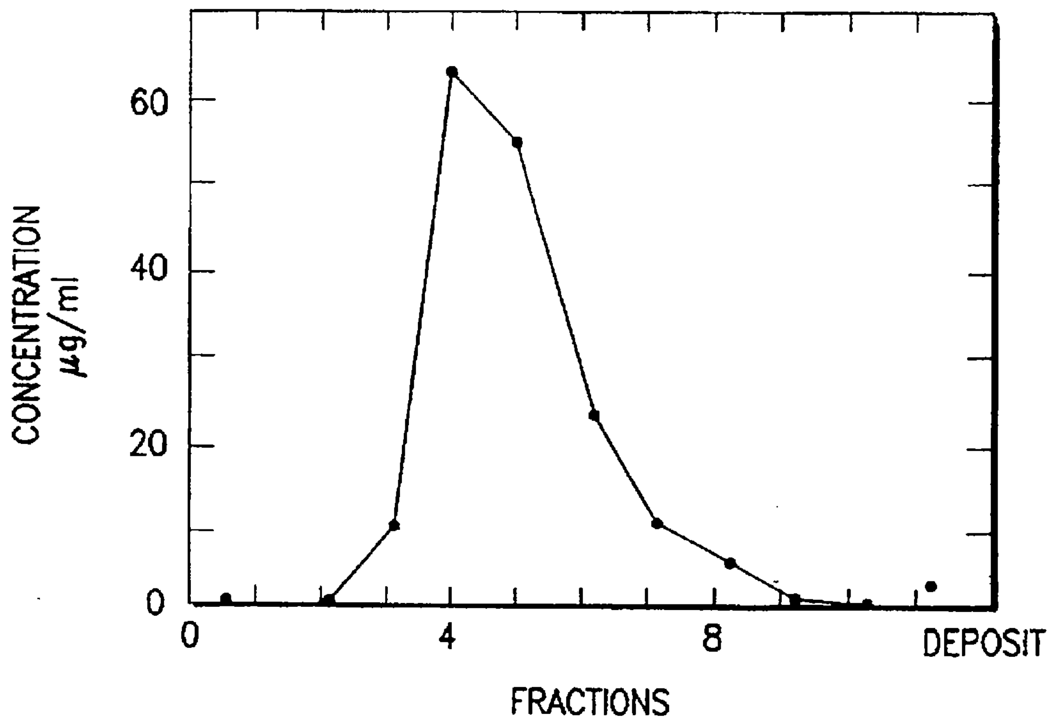



FIG. 4

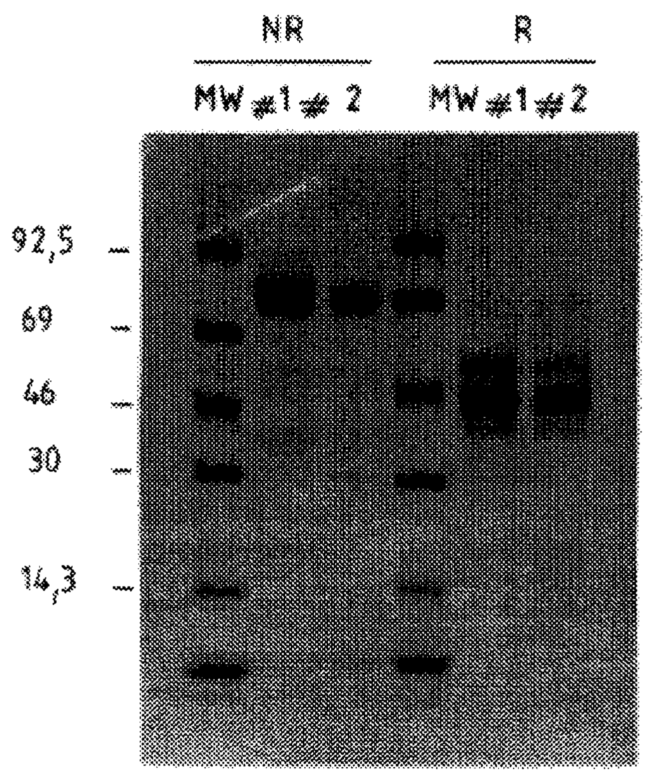



FIG.5

# PRODUCTION OF SUBUNITS OF SOLUBLE T CELL RECEPTORS BY CO-TRANSFECTION

## BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to soluble T receptors and more particularly to secreted forms of soluble T receptors (sTR) VαC/α/VβC/β, VγCγ/VδCδ or VαCδ/VβC/γ and to their diagnostic and therapeutic applications.

2. Description of the Related Art

T lymphocytes are capable of recognizing, in a highly specific manner, myriads of antigens (Ag); this is by means of extremely diverse surface structures belonging to the superfamily of immunoglobulins (Ig), the T receptors (TR).

In man and in mice, most T lymphocytes in adults express sTR consisting of 2 variable glycoprotein sub-units called α and β. Like the Ig heavy and light chains, these subunits contain an amino-terminal variable (V) domain and a carboxy-terminal constant (C) domain and are, in addition, very generally covalently associated with each other via an interchain disulphide bridge. The nature of the antigens recognized by the αβ T receptor is relatively well established: they are complexes formed by an oligopeptide antigen (derived from the intracellular degradation of endogenous or exogenous proteins) closely associated with the polymorphic gene products situated in the so-called class I or II major histocompatibility complex (MHC). The interaction between the αβ T receptor and the MHC/Ag complexes is conventionally reinforced by so-called coreceptor or accessory molecules (CD4 and CD8), which recognize conserved portions of the class II and I MHC molecules respectively.

Another subpopulation of T lymphocytes which can be distinguished by the nature of the genes (γ and δ) encoding these T receptors has more recently been described. Contrary to the αβ T lymphocytes, the antigenic specificity of the γδ T cells still remains unclear. Based on the relative homology of the primary sequences of the αβ and γδ chains of the T receptor, some have predicted a structural similarity of the ligands for these receptors. In agreement with this hypothesis, a fraction of the γδ T lymphocytes was found to be directed against molecules structurally similar or identical to the products of the MHC conventionally recognized by the αβ T lymphocytes. However, there are also several examples of recognition by this T subpopulation of molecules of more distant structure, such as stress proteins or certain activating molecules such as CD48.

The present inventors have sought to generate "soluble" (secreted) forms of the γδ T receptor, which could be used (like the Ig's) as probes permitting the isolation, localization and possibly the purification of specific ligands.

Moreover, such soluble T receptors also have a number of clinical applications. Traunecker et al. (1989, Inununol. Today 10:29) have reported attempts to produce soluble T receptors which consisted in removing the transmembrane (TM) portion of the α chains or β chains by introducing a translational termination codon upstream of the sequences encoding the TM region which proved unsuccessful, no secretion having been detected.

Following these initial failures, other strategies were then adopted. In most cases, the principle consisted in constructing chimetic proteins comprising the V, or V and C regions of the α and β subunits, joined to the C regions of immunoglobulins or to anchors of the glycosyl phosphatidylinositol (GPI) type. In the case of the TR/Ig fusion proteins, the main problem proved to be the sometimes predominant secretion of monomeric or homodimeric forms. In addition, the αβ sTR heterodimeric forms sometimes exhibited significant structural differences with the membrane forms; in particular, the 2 α and β chains were very generally non-covalently associated. This could consequently have effects on the overall structure and the fine antigenic specificity of such chimeric molecules. In the case of "lipidated" T receptors (anchored to the membrane by a GPI sequence), a sometimes quite high proportion of covalently associated αβ heterodimers could be obtained. However, the main disadvantage of this technique was the need for an enzymatic treatment (with phospholipase C), in order to liberate the T receptors in the medium, and therefore a production which is costly and of low yield. A procedure for producing so-called monochain T receptors, consisting in joining a Vα domain to a Vβ domain via a peptide bridge, has been proposed more recently. However, the use of this technique proved to be delicate. In particular, it assumed the introduction of a large number of mutations in certain hydrophobic zones of the V regions normally masked on the native protein, in order to render these monochain T receptors hydrosoluble.

All the examples of the production of soluble forms of T receptors described in the literature, in all cases in hybrid form, have shown an extreme variability of efficiency from one chain combination to another.

## SUMMARY OF THE INVENTION

The present inventors have discovered that soluble T receptors could be easily obtained and with a high yield, regardless of the combination of chains used, by means of a process consisting in producing DNA molecules encoding each of the constituent T receptor submits from which the transmembrane portion has been deleted, and in co-transfecting these DNAs into a host cell.

The subject of the present invention is also a process for producing soluble T receptors, wherein the DNA sequences encoding each of the constituent T receptor submits, from which the transmembrane portion of the T receptor has been deleted, are co-transfected into a host cell.

According to the invention, VαC/α/VβCβ soluble T receptors are produced by co-transfecting, into a host cell, DNA sequences encoding the α and β submits of the Tαβ receptor from which the transmembrane portion of the Tαβ receptor has been deleted.

VγCγ/VδCδ soluble T receptors are also produced by co-transfecting, into a host cell, DNA sequences encoding the γ and δ submits of the Tγδ receptor from which the transmembrane portion of the Tγδ receptor has been deleted.

VαCγ/VβCδ and VαCδ/VβC/γ heterodimeric soluble T receptors are further produced, in which the constituent subunits are associated via a covalent bond, by co-transfecting, into a host cell, DNA sequences encoding the Cγ and Cδ domains of the γ and δ subunits of the Tγδ receptor from which their transmembrane portion has been deleted, fused respectively to the DNA sequences encoding the Vα and Vβ domains of the α and β subunits of the Tαβ receptor in order to obtain VαCγ/VβCδ receptors, or fused respectively to DNA sequences encoding the Vβ and Vα domains of the β and α subunits of the Tαβ receptor in order to obtain VαCδ/VβCδ receptors.

VγCγ/VαCδ hybrid soluble T receptors are also produced by co-transfecting, into a host cell, DNA sequences encoding the γ subunit of the Tγδ receptor from which its trans-

membrane portion has been deleted, with the DNA sequences encoding the $C\delta$ domain of the $\delta$ subunit fused to the DNA sequences encoding the $V\alpha$ domain of the $\alpha$ subunit of the $T\alpha\beta$ receptor. This construction is particularly advantageous and is based on the fact that certain $V\alpha$ genes can be used either by $\alpha\beta$ clones, or by $\gamma\delta$ clones.

Advantageously, the DNA sequences of the $V\delta2$ and $V\gamma9$ genes are used, for the constructions of the soluble T receptors of the invention, for the variable parts.

It may however be advantageous to produce $V\gamma C/65$ $/V\delta C\delta$ receptors using a $V\gamma9$ DNA sequence on the one hand, and by replacing the $V\delta2$ DNA sequence by other $V\delta$ DNA sequences for the same reasons as those mentioned above for the construction of the $V\gamma C\gamma/V\alpha C\delta$ hybrid receptor. This construction makes it possible to obtain anti-$\alpha$ antibodies, or antibodies directed against $V\delta$'s distinct from $V\delta2$.

Conversely, it is also possible to conserve the $V\delta2$ DNA and to replace the $V\gamma9$ DNA sequence with other $V\gamma$ DNA sequences, in order to obtain anti-$V\gamma$ antibodies.

The invention also encompasses these embodiments of $V\gamma C\gamma/V\delta C\delta$ soluble T receptors.

It should be noted that several $V\delta$ segments (especially $V\delta1$) can be considered as $V\alpha$'s, in the sense that they can be equally used by the $\alpha$ or $\delta$ chains of the T receptor. Thus, it can be considered that the examples of receptors produced in soluble form, which are provided here, demonstrate especially the usefulness of the process within the framework of the generation of monoclonal antibodies directed not only against the $\gamma$ and $\delta$, but also $\alpha$, variable regions.

Advantageously, the deletion of the transmembrane portion of the constituent T receptor submits is carried out by introducing a translational termination codon upstream of the sequences encoding the transmembrane portion of these submits, especially by P.C.R. (Polymerase Chain Reaction) directed mutagenesis.

The DNA sequences are genomic DNA or cDNA sequences.

Preferably, the co-transfection is carried out into eukaryotic cells, especially hamster ovary cells (CHO).

The subject of the invention is also a fusion protein formed between a soluble T receptor and a peptide sequence, the peptide sequence being constitutive of a peptide or of a protein, the fusion protein being obtained by fusing the DNA sequence encoding the peptide or the protein to one of the chains or to the two chains of DNA encoding the submits of a T receptor from which their transmembrane portion has been deleted, followed by a co-transfection of the DNA sequences thus fused into a host cell.

Advantageously in this case, the peptide sequence is that of interleukin-2 (IL-2).

The subject of the invention is also human or animal polyclonal or monoclonal antibodies directed against a soluble T receptor obtained by the process of the invention or an sTR-IL2 fusion protein as defined above.

The monoclonal antibodies according to the invention can be prepared according to a conventional technique. To this effect, the soluble T receptors, optionally fused with interleukin-2 or another protein, can be coupled if necessary to an immunogenic agent, such as tetanus toxoid, via a coupling agent such as a his diazotized benzidine.

The present invention also encompasses the fragments and the derivatives of monoclonal antibodies according to the invention. These fragments are especially $F(ab')_2$ fragments which can be obtained by enzymatic cleavage of the antibody molecules with pepsin, the Fab' fragments which can be obtained by reducing the disulphide bridges of the $F(ab')_2$ fragments and the Fab fragments which can be obtained by enzymatic cleavage of the antibody molecules with papain in the presence of a reducing agent. These fragments, as well as the Fc fragments, can also be obtained by genetic engineering.

The derivatives of monoclonal antibodies are for example antibodies or fragments of these antibodies to which markers such as a radioisotope are linked. The derivatives of monoclonal antibodies are also antibodies or fragments of these antibodies to which therapeutically active molecules are linked.

The subject of the invention is also hybridomas producing monoclonal antibodies specific for the peptide sequence described above. These hybridomas can be obtained by the conventional techniques of cell fusion between spleen cells activated in vitro by the antigen or obtained from an animal immunized against the peptide sequence of the invention and cells from a myelomatous line.

The subject of the invention is also a diagnostic composition comprising a soluble T receptor obtained by a process according to the invention or an sTR-peptide sequence, especially sTR-IL2, fusion protein as defined above, or alternatively a monoclonal antibody according to the invention.

The diagnostic composition according to the invention can be used for the typing of cellular specificities linked to the T receptor. Indeed, a soluble T receptor can be used as such. However, because of the probably weak affinity of the latter for its specific ligand, it is advantageous to couple the soluble T receptors to a support, in order to increase their avidity by increasing their valency.

The support may consist of any support traditionally used, such as organic or magnetic beads.

Such supports are for example plastic plates used for the ELISA tests on which the soluble T receptor is attached in the same manner as immunoglobulins, tosyl-activated magnetic beads, for example those marketed by Dynal, Oslo, Norway, or alternatively AFFIGEL type activated gels such as those marketed by BIORAD.

The coupling techniques are those conventionally used and indicated by the distributor for the supports commercially available.

These methods may consist in a chemical coupling or by means of monoclonal antibodies directed against the soluble T receptors in question, the latter being themselves coupled to the support by chemical coupling.

Advantageously, the diagnostic compositions comprise a fused protein as described above, consisting of a soluble T receptor and an antigenic determinant against which specific antibodies are available.

Such diagnostic compositions can be used for the typing of cellular specificities not detected by conventional serological techniques.

The diagnostic composition according to the invention may also comprise monoclonal antibodies as defined above, and preferably a panel of monoclonal antibodies directed against the V and C portions of the chains of the T receptors obtained by immunizing animals against the soluble T receptors obtained according to the invention, previously purified.

In order to improve the efficacy of the immunizations, it is also possible to inject sTR-IL2 fusion proteins as defined above.

Such a diagnostic composition can be used especially for the detection of mono- or oligoclonal proliferations, such as those encountered in T leukaemias for example.

According to the invention, the diagnostic composition is brought into contact with a biological sample, for example a blood sample containing pathological T lymphocytes, and the complex formed with the ligand specific for the T receptor and the soluble T receptor or the fusion protein comprising the soluble T receptor and an antigenic determinant or the complex formed by the monoclonal antibodies according to the invention and the soluble T receptor or the soluble T receptor-IL2 fusion protein against which they are specifically directed, is detected.

These processes can be based on an RIA, RIPA or IRMA type radioimmunological method, or an immuno-enzymatic method of the WESTERN-BLOT type on strips or of the ELISA type.

For the implementation of these processes of detection, unlabelled cold molecules or molecules labelled by means of a suitable marker which may be biotin or its derivatives, an enzyme such as peroxidase, a fluorescent marker such as fluorescein, a radioactive marker and the like, are used.

These in vitro diagnostic processes comprise for example the following steps:

depositing a determined quantity of a composition containing a soluble T receptor, a soluble receptor fused with an antigenic determinant or a monoclonal antibody according to the invention directed against the soluble T receptor or the soluble T receptor-Interleukin 2 fusion protein according to the invention, in the wells of a microtitre plate or on another support such as beads or a nitrocellulose membrane,

depositing, in the wells, the biological sample to be tested, or incubating the latter with the beads or the membrane, in the presence of saturating agents or after prior saturation of the activated supports,

after incubating and rinsing the microplates or the beads, depositing in the wells or incubating with the beads a system for revealing the soluble T receptor-ligand complex which may have formed.

The kits for implementing the diagnostic process of the invention comprise:

at least one diagnostic composition according to the invention,

reagents for preparing a medium suitable for producing a complex between the ligand(s) which may be present in a biological sample,

one or more optionally labelled reagents capable of reacting with the complex formed.

The subject of the invention is also a therapeutic composition characterized in that it comprises a soluble T receptor obtained according to the process of the invention or a fusion protein as defined above, especially an sTR-IL2 according to the invention.

Such a therapeutic composition is useful especially in the treatment of pathological processes in which a paucicIonal proliferation of T lymphocytes is observed, such as T leukaemias or lymphomas and certain autoimmune diseases.

It is preferably administered by injection in an appropriate vehicle.

The administration of this therapeutic composition has a double purpose. It permits, on the one hand, the induction of an anti-idiotypic immuno response, resulting, in this case, in the active and selective removal of the cells carrying these idiotypes, and, on the other hand, the blocking, by competition, of the recognition of autologous antigens in the case of auto-immune proliferations.

Advantageously, the therapeutic composition according to the invention comprises a heterodimeric soluble T receptor as defined above, optionally carried by a fusion protein.

The therapeutic composition according to the invention may also comprise a monoclonal antibody according to the invention, optionally coupled to a therapeutically active molecule, for example a cytotoxic molecule, or a monoclonal antibody fragment or derivative as defined above.

Such a composition permits the direct removal of mono- or oligoclonal cells encountered in certain types of T leukaemias.

## BRIEF DESCRIPTION OF THE DRAWINGS

The production of soluble T receptors in the case of TRγδ will be described in detail below with reference to the accompanying figures in which:

FIGS. 1 and 1B represent products of assembly of the γ and δ genes. The sequences of the 5' and 3' primers used to amplify the cDNAs permitting the production of the soluble Tγδ receptors (γs and δs cDNA) are represented above and below the γ and δ cDNAs respectively. The positions of the termination codons are represented in bold characters. The grey parts in 3' of the γ and δ cDNAs correspond to the hydrophobic transmembrane (TM) regions. FIG. 1 shows the sequences SEQ ID NO:1–SEQ ID NO:15 as depicted on the attached sequence listing.

FIGS. 2A and 2B represent the corresponding nucleotide and peptide sequences of the soluble δ and γ chains of the clone used for the construction of the soluble T receptor described above. In particular, FIG. 2A shows the sequences SEQ ID NO:16 and SEQ ID NO:17 and FIG. 2B shows the sequences SEQ ID NO: 18 and SEQ ID NO:19 as depicted on the attached sequence listing.

FIG. 3 represents the results of the tests for detection of sTRγδ by the IRMA technique in medium packaged from CHO cells transfected with γs/δs.

SN represents the supernatant from the culture of the CHO cells, transfected with a non-pertinent cDNA (C) or with the cDNAs of the soluble γ and δ subunits according to the invention (sγδ).

The monoclonal antibodies giving a significant radioimmunological signal are represented as bold rectangles.

FIG. 4 represents the titration in soluble TR activity expressed in μg/ml, as attested by the IRMA test (sandwich 7B6/TiVδ2), of the fractions eluted from an affinity column coupled with the anti-Vγ 7B6 antibody (marketed by Immunotech), onto which have been applied about 500 ml of supernatant from the culture of γδsFS-CHO cells.

FIG. 5 represents the SDS-PAGE analysis of fractions positive for the soluble TR activity, as attested by the IRMA test (sandwich 7B6/TiVδ2), of the fractions eluted from an affinity column coupled with the anti-Vγ9B6 antibody.

Two independent preparations (#1 and #2) were analysed under non-reducing (on the left) and reducing (on the right) conditions. MW=molecular weight markers.

## DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

### EXAMPLE 1

#### 1. Construction And Expression of the γδs Genes For the sTRs

The γδs G 115 human lymphocyte clone (whose nucleotide and peptide sequences corresponding to the soluble δ

and γ chains are represented in FIG. 2, in A and B respectively) expressing T V9JPCIγ/V2D3J1Cδ receptors was used for the construction of the γδs genes and the expression of the soluble T receptors.

This clone was used for several reasons of which the main ones are:

- the great majority of the γδ T receptors of peripheral blood human leucocytes comprise similar V(D)J regions such that the structural and functional results obtained with the soluble form of the specific TR used can be easily applied to the TR expressed by a large proportion of γδ cells,
- monoclonal antibodies specific for the Cγ, Cδ, Vγ9 and Vδ2 regions are easily available and can be used to monitor the production and the purification of the soluble TR molecules,
- unlike most γδ Vδ1-positive human T lymphocytes, the γ and δ chains of the T receptors of the G 115 clone are covalently linked by a disulphide bridge which highly stabilizes the molecule after its secretion into the medium,
- the antigenic specificity of the G 115 clone is fairly well known. In particular, this clone kills the cells of a Burkitt's lymphoma (called Dadi) and also recognizes an antigen present in water-soluble extracts of *Mycobacterium tuberculosis.*

The G 115 clone, obtained from Tγδ lymphocytes derived from human peripheral blood leucocytes, was maintained in an RPMI 1640 medium containing 8% human serum, 2 mM L-glutamin and 150 BRMP (Biological Response Modifier Program) units of IL2 and stimulated for one week out of two with 0.5 μg/ml of leucoagglutinin (Pharmacia, France), irradiated peripheral blood leucocytes and irradiated and EBV-transformed B lymphoblasts.

After two washes in phosphate buffered saline solution, $5\times10^6$ cells were lysed on ice in a Tris-HCl buffer (80 mM, pH 7.5) containing 100 mMNaCl, 5 mM EDTA and 0.5% by weight of Triton X100. After centrifuging, the supernatant was harvested and mixed with an equal volume of phenol at 65° C. The RNA was extracted by a phenol/$CHCl_3$ treatment, precipitated in 2.5 volumes of ethanol and solubilized in 40 μl of 10 mM Tris/1 mM EDTA). 5 μl of total RNA were reverse-transcribed for 1 hour at 37° C. by means of a 3'-phosphated primer containing translational termination codons upstream of the hydrophobic transmembrane region of the γ and δ genes, after the $Lys^{247}$ and $Gln^{274}$ codons, as represented in FIG. 1, at a concentration of 50 pM, the four dNTPs at a concentration of 1 mM each and 200 units of mouse mammary tumor virus reverse transcriptase (MMTV) (Boehringer Mannheim, Germany), in a final volume of 25 μl. 1.75 μl of a mixture for PCR (containing 13 mM Tris-HCl (pH 8.2), 66 mM KCl, 2 mM $MgCl_2$, 2 U of Taq polymerase (Boehringer) and 50 pM of 5'-phosphated primer represented in FIG. 1 were added to the material obtained by reverse transcription and 30 amplification cycles (94° C.—1 min, 45° C.—1 min, 72° C.—1 min) were carried out. The amplified DNA was purified after electrophoresis on a low melting point agarose gel and cloned into a plasmid Bluescript SK+ (Stratagene, La Jolla, Calif.) digested with SmaI. The sequencing was carried out using a system of double-stranded template according to the procedure provided by the supplier of the USB Sequenase kit. The fragments were cloned into an expression vector pKCR6 (Matrisian et al., Proc. Natl. Acad. Sci. USA. 83:9413) digested with EcoRI.

The plasmid DNA was then introduced into DHFR (dihydrofolate reductase)-negative hamster ovary cells DUKX-B11, cultured in RPMI 1640 medium, supplemented with 8% foetal calf serum, 2 mM L-glutamin, thymidin, adenosin and deoxyadenosin at 10 μg/ml each, by the calciumphosphate precipitation technique (Wiglet et al., 1979 Cell. 16:777). The DHFR-positive cells were selected by culturing the transfected cells for three weeks in RPMI medium, supplemented with foetal calf serum and n-glutamin (2 mM) without nucleosides. The stable transfectants were then cloned by the limiting dilution technique.

## 2. Detection, Purification And Characterization of the Soluble Tγδ Receptors

a) Detection of the soluble T receptors

The monoclonal antibodies used for the detection of the soluble TRs were labelled with $^{125}I$ by the Iodogen method (Fraker et al., 1978, Biochem.-Biophys. Res. Commun. 80:849). The T receptors were detected by a sandwich immunoradiometric assay (IRMA) by means of pairs of monoclonal antibodies specific for the γ and δ chains.

Immulon-1 microtitre plates (Dynatech, Marnes, France) were coated for 90 min at 37° C. with 50 μl of Y102 (or 7B6) monoclonal antibody at 40 μg/ml in a phosphate buffered saline solution. After removal of the antibody, the unbound sites were saturated with a phosphate buffered saline solution containing 0.5% bovine serum albumin for 1 hour at room temperature. The samples to be analysed were then added in an amount of 40 μl at the same time as 10 μl of labelled TiVδ2 monoclonal antibody. After incubating for 90 min at 37° C., the wells were rinsed four times with 100 μl of a phosphate buffered saline solution supplemented with bovine serum albumin.

The bound radioactivity was measured in a γ scintillation counter. The following set of antibodies was used to measure the secretion of soluble TRγδ by the IRMA technique: anti-Vγ9 (Y102, B37, 7B6), anti-Cγ (B121) and anti-Vδ2 (TiVδ2) antibodies (Miossec et al., 1989, J. Exp. Med. 171:1171). A monoclonal antibody specific for IL2 was also used as negative control.

With the various combinations of antibodies, no signal was observed with the supernatants of non-transfected hamster ovary cells (CHO), of cells transletted with a non-pertinent cDNA or of cells transfected either with a truncated γs cDNA or a truncated δs cDNA (FIG. 3). But the soluble γδ hetero-dimers were clearly detected by IRMA (radioimmunological assay) in the supernatants of CHO cells co-transfected with soluble γ and soluble δ assembly products (γδsFS-CHO) when pairs of antibodies specific for Vδ2/Cγ or Vδ2/Vγ9 were used (FIG. 3), which suggests that the soluble TR molecules secreted by the γδF5-CHO cells were predominantly heterodimers.

b) Purification of the soluble T receptors

10 mg of Y102 or 7B6 monoclonal antibody (anti-Vγ9) were covalently linked to a matrix of activated agarose beads (Affigel, Biorad, Richmond, Calif.) according to the instructions of the supplier.

The culture supernatants were applied to an affinity column at a rate of 30 ml/h at 4° C. After washing with a phosphate buffered saline solution, the bound material was eluted with a 0.2M glycine buffer (pH 2.5). The eluted fractions were neutralized all at once with 1M $Na_2HPO_4$.

The fractions positive for the soluble TR activity as attested by the IRMA test were combined, dialysed overnight against distilled water and concentrated by evaporation.

Soluble TR samples were prepared in a buffer for gel electrophoresis with or without reducing agent, separated by

SDS-PAGE and transferred onto a nitro-cellulose membrane in accordance with the recommendations of the supplier. After saturating the unbound sites with a blocking buffer (dried skimmed milk and Tween 20), the fingerprints obtained were incubated in the presence of primary antibody (hybridoma supernatant diluted one-third with the blocking buffer) for 2 hours at room temperature. After washing, an anti-Ig-horseradish peroxidase conjugate was added, and the incubation continued for another 2 hours. The bound antibodies were revealed with diaminobenzidine (1 mg/ml), $H_2O_2$ and $CoCl_2$.

In a typical preparation, 3.3 mg (calculated using a coefficient for 1% extinction of 1.5, as calculated for the immunoglobulins) of affinity-purified γδ TRs were treated with Vibrio cholerae neuraminidase (Boehringer Mannheim) in 1 ml of buffer containing 50 mM sodium acetate, 150 mM NaCl and 4 mM $CaCl_2$ at pH 5.5 for 1 hour at 37° C.

Under these conditions, the reaction was estimated to be complete by determining control assays for digested samples by isoelectric focusing in IEF 3-9 PhastGel medium (Pharmacia).

After dilution with a 0.1M sodium phosphate buffer, pH 7.3, the sample was concentrated by means of a centripep column at 30,000 revolutions (Amicon) before protelysis.

The neuraminidase-treated γδ receptors were digested at 37° C. for 30 minutes with papain (Worthington) at an enzyme/substrate ratio of 1/500 in the presence of 1.5 mM 2-mercaptoethanol and 1.25 mM EDTA. The reaction was completed by addition of N-ethylmaleimide.

These conditions were sufficient to completely eliminate the interchain disulphide bridge as attested by SDS-PAGE analysis under non-reducing conditions. Higher enzyme/substrate ratios and/or longer incubation times provided no proof of an additional protein cleavage. The reaction medium was then applied to a Zorbax CF-250 size-exclusion chromatography column (DuPont - New England Nuclear) which made it possible to obtain, after elution, the T receptor treated with papain and neuraminidase in the form of a single peak at about 65 kDa compared with 75 kDa for the native protein. No sign of chain dissociation was apparent.

After concentrating on a centripep, the material described above was incubated overnight at 37° C. in the presence of endoglycosidase F and N-glycosidase F (Boehringer Mannhein) under non-denaturing conditions (0.1M sodium phosphate buffer, pH 7.3), as recommended by the manufacturer. A final purification was carried out by means of a Mono Q high-performance anion-exchange chromatography column (Pharmacia).

The total yield from 3.3 mg of affinity-purified T receptor was 1.1 mg or about 34%.

The material eluted from the anti-Vγ9 column consisted essentially of γδ heterodimers since it was precipitated by monoclonal antibodies specific for Vδ2. In addition, an SDS-PAGE analysis under reducing and non-reducing conditions showed that these heterodimers were linked by a covalent bond.

Indeed, under non-reducing conditions, a diffuse principal band having an apparent molecular weight of 75–80 kD was observed, which separated under reducing conditions into two predominant components of 42 and 44 kD and two minor components of 50 and 39 kD. Identical patterns were obtained with material precipitated in stages with anti-Vγ9 and anti-Vδ2 monoclonal antibodies. By means of monoclonal antibodies generated against this soluble receptor (monoclonal antibodies 360 and 389, cf. below), it was possible to show by the Western-blot technique that the 50 kD and 44 kD bands corresponded to the γ chain, and that the 42 and 39 kD band corresponded to the δ chain. The differences in the sizes of the soluble γ and δ species were due to the different degrees of N-glycosylation, as subsequently specified.

### 3. Production And Properties of Monoclonal Antibodies Directed Against the Soluble T Receptors of the Invention

a) Generation of monoclonal antibodies directed against soluble forms of γδ TR after immunization of mice against soluble γδ TRs:

BALB/c mice were immunized with soluble γδ T receptors, in accordance with the following procedure: on day 1, 50 μg of protein in 500 μl of emulsified complete Freund's adjuvant at 50% in 0.9% NaCl were subcutaneously injected at four different points. On day 25, the same procedure was repeated in incomplete Freund's adjuvant. A booster was made by 3 intraperitoneal injections on days 50, 51 and 52, by means of 15 μg of protein each in 250 μl of 0.9% NaCl. Splenocytes harvested on day 53 were fused with X63 Ag 8653 myeloma. Hypoxanthine/aminopterin/thymidin-resistant colonies were screened by a radioimmunological assay (RIA) by means of an iodine-labelled soluble T receptor, in accordance with the IODOGEN method.

To this effect, 96-well microtitre plates coated with avidin (Immunotech) were incubated with biotinylated anti-mouse goat immunoglobulins (GAMIG, Immunotech) in PBS, BSA, $NaN_3$ overnight at 4° C., and then washed 3 times in Tween PBS. 100 μl ($10^5$ cpm) of radiolabelled soluble T receptors were incubated for 2 hours at room temperature and washed 3 times in PBS-Tween. The bound radiolabelled soluble T receptors were assayed by γ counting.

Nine monoclonal antibodies recognizing all or part of the human γδ T lymphocytes were obtained from an immunized mouse spleen, 2 anti-Vγ9 antibodies (292 and 360), 2 anti-Vδ2 antibodies (1 and 389), 1 γδ pan anti-body (510) and 4 antibodies directed against γδ sub-populations (49, 60, 103 and 515).

b) Reactivity of anti-soluble TR monoclonal antibodies towards mono- and polyclonal human lymphold lines:

Monoclonal antibodies having produced an RIA signal were then tested by immunofluorescence to determine their ability to recognize T receptors linked to the membranes of the G9 clone. The fine specificity of these monoclonal antibodies was finally studied by screening their reactivity towards T lymphocyte clones and lines whose T receptor phenotype was known.

From a single fusion experiment, the supernatants of 16 colonies (3% of the inoculated wells) gave a positive RIA signal and among them, eleven contained monoclonal antibodies recognizing the G9 clone in an indirect immunofluorescence assay. The specificity of 7 monoclonal antibodies was measured by flow cytometric analysis.

Three monoclonal antibodies (52, 106 and 510) were directed against a determinant which was common to all the Tγδ receptors but not to the Tαβ receptors. Two monoclonal antibodies (292 and 360) were specific for T receptors comprising the Vγ9 region and two monoclonal antibodies (1 and 389) for T receptors comprising the γδ2 region. No precise specificity could be attributed to the remaining monoclonal antibodies (49, 60, 103 and 515) which recognized subpopulations of γδ lymphocytes but whose reactivity could not be correlated with the presence of a particular V region of T receptor (Table I below).

It should be noted that all the monoclonal antibodies were capable of recognizing non-reduced soluble T receptors in Western-blot analyses, and several also reacted with γ or δ species isolated after reduction (Table II), unlike most V-specific monoclonal antibodies generated against native T receptors (linked to membranes). In agreement with attributions of specificity deduced from flow cytometric experiments, the monoclonal antibodies 389 and 360 recognized various species (molecular mass 39–42 kDa and 44–50 kDa, respectively), which could correspond to the δ and γ chains respectively. In addition, since the γδ52 and 510 pan monoclonal antibodies, and the antibody 389 specific for Vδ2 reacting with the same species of 39–42 kDa, this indicating that the monoclonal antibodies 52 and 510 were directed against the Cδ region (Table I).

In order to facilitate and to permit the oriented integration of the complementary DNAs encoding the soluble gamma and delta chains in the eukaryotic system expression vector pKCR6, a DNA fragment previously cloned between the XbaI and SalI sites of the vector pKCSRα was introduced between the KpnI sites of this vector.

The digestion of the vector pKCR6 thus modified by the XhoI and XbaI enzymes liberated these two sites and permitted an oriented cloning, the XhoI site being situated between 5' of the coding sequence and the XbaI site in 3'.

b) Generation of a complementary DNA encoding a soluble Vγ8 chain

b1) PCR cloning of a soluble Vγ8 chain

The RNA used for this cloning is obtained from a Tγδ clone.

TABLE 1

Circulating cytometric analysis of clones of Tγδ by means of an anti-sTR monoclonal antibody. The phenotype of the T lymphocyte clones was determined by labelling with Trγa (anti-Bγ9), TiVδ2) and A13 (anti-Vδ1) antibodies; NR (not carried out).

| Clones | Vγ9 | Vδ2 | Vδ1 | 510 | 106 | 292 | 360 | 1 | 389 | 49 | 60 | 103 | 515 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| G9 | + | + | − | + | + | + | + | + | + | + | + | + | + |
| M39 | + | + | − | + | NR | + | + | + | + | − | + | + | + |
| G12 | + | + | − | + | NR | + | + | + | + | − | + | + | + |
| Tγ3 | + | + | − | + | + | + | + | + | + | − | + | + | + |
| Tγ6 | + | + | − | + | + | + | + | + | + | − | + | + | + |
| Tγ11 | + | + | − | + | + | + | + | + | + | − | + | + | + |
| Tγ12 | + | + | − | + | + | + | + | + | + | − | + | + | + |
| Tγ15 | + | + | − | + | + | + | + | + | + | − | + | + | + |
| Tγ30 | + | + | − | + | + | + | + | + | + | − | + | + | + |
| G93 | + | + | − | + | + | + | + | + | + | − | − | − | − |
| T14 | − | + | − | + | NR | − | − | + | + | − | + | + | + |
| F11 | + | − | + | + | NR | + | + | − | − | − | − | − | − |
| M7 | − | − | + | + | NR | − | − | − | − | − | − | − | − |
| M8 | − | − | + | + | + | − | − | − | − | − | + | − | − |

TABLE II

Results of the Western-blot analyses of soluble T receptors by means of anti-sTR antibodies. The apparent molecular mass (in kDa) of the species recognized by each antibody is presented. (*NR = no reactivity; R = reactivity)

| | Western-blot analysis | | |
|---|---|---|---|
| Hybridoma | NR* | R* | Specificity |
| 52 | 80 | 39/42 | pan δ |
| 106 | 80 | — | pan γδ |
| 510 | 80 | 39/42 | pan δ |
| 1 | 80 | — | Vδ2 |
| 389 | 80 | 39/42 | Vδ2 |
| 292 | 80 | — | Vδ9 |
| 360 | 80 | 44/50 | Vδ9 |
| 49 | 80 | — | γδ subpopulations |
| 60 | 80 | — | γδ subpopulations |
| 103 | 80 | 39/42 | δ subpopulations |
| 515 | 80 | 39/42 | δ subpopulations |

## EXAMPLE 2

1. Construction of Other γδ Soluble T Receptors

Other γδ soluble receptors were prepared as described below, after modification of the multiple cloning site of the expression vector pKCR6.

a) Modification of the multiple cloning site of the expression vector pKCR6

The oligonucleotide primer used for the synthesis of the first complementary DNA strand is the following:

5' GGG TTA CTG CAG CAG TAG TGT ATC 3' (SEQ ID NO:1)

The amplification of this cDNA was carried out by means of the oligonucleotide described above used as antisense primer and a sense primer containing a site for the XhoI restriction enzyme upstream of the translational initiation codon. The sequence of this oligonucleotide is the following:

5' CCC TCG AGA TGC TGT TGG CTC TAG CTC 3' (SEQ ID NO:2)

The DNA fragment obtained at the end of this amplification was cloned into the vector pBS-SK opened by the SmaI restriction enzyme and then sequenced. The sequence obtained is in conformity with that described in the literature (Cell. (1986) 45:237–246) with the exception of the joining sequence involving the Jγ1 segment:

Vγ8 N Jγ1 TGT GCC ACC TGG GAC AGT CAT TAT TAT AAG AAA CTC TTT (SEQ ID NO:3)

b2) Integration into the expression vector and transfection into eukaryotic cells

The cDNA fragment encoding a soluble Vγ8 chain was extracted from the vector pBS-SK after digestion with the

restriction enzymes XhoI and XbaI and integrated into the modified expression vector pKCR6 described in a) digested with the same enzymes.

The vector thus obtained was co-transfected in combination with the expression vector containing the cDNA encoding the soluble Vδ2 chain.

The procedure for transfection, screening of the producing clones and purification of the soluble TCRs produced is analogous to that described above for the production of soluble Vγ9 Vδ2 TCR.

c) Generation of a complementary DNA encoding a soluble Vδ3 chain

c1) PCR cloning of a soluble Vδ3 chain

The RNA used for this cloning is obtained from a Tγδ clone.

The nucleotide primer used for the synthesis of the first complementary DNA strand is the following:

5' GGG TTA CTT CTC GGT ATG AAC TAT GGC 3' (SEQ ID NO:4)

The amplification of this cDNA was carried out by means of the oligonucleotide described above used as antisense primer and a sense primer containing a site for the XhoI restriction enzyme upstream of the translational initiation codon. The sequence of this oligonucleotide is the following:

5' GAC TCG AGA AAA GAT GAT TCT TAC TGT GGG 3' (SEQ ID NO:5)

The DNA fragment obtained at the end of this amplification was cloned into the vector pBS-SK opened by the SmaI restriction enzyme and then sequenced. The sequence obtained is in conformity with that described in the literature (j. Exp. Med. (1989) 169:393–405) with the exception of the joining sequence involving the Dδ2, Dδ3 and Jδ1 segments:

Vδ3 N Dδ2 N Dδ3 ACT TAC TGT CCT T TT TCC CGG CTC T TG GGG G AC ACC Jδ1 GAT AAA (SEQ ID NO:6)

c2) Integration into the expression vector and transfection into eukaryotic cells

The cDNA fragment encoding a soluble Vδ3 chain was extracted from the vector pBS-SK after digestion with the restriction enzymes XhoI and XbaI and integrated into the modified expression vector pKCR6 described in a) digested with the same enzymes.

The vector thus obtained was co-transfected in combination with the expression vector containing the cDNA encoding the soluble Vγ9 chain.

The procedure for transfection, screening of the producing clones and purification of the soluble TCRs produced is analogous to that described above for the production of soluble TCR Vγ9 Vδ2.

d) Generation of a complementary DNA encoding a soluble Vδ1 chain

The DNA complementary to a total Vδ1 Cδ chain cloned into the vector pBS-SK between the SalI and BamHI restriction sites was used.

This fragment was sequenced completely and exhibits no variation compared with the sequence described in the literature (Eur. J. Immunol. (1989) 19:1545–1549) with the exception of the joining sequence involving the Dδ2 and Jδ1 segments:

Vδ1 Dδ2 N TGT GCT CTT GGG GAC TTC CTA AAG GGT TCA GGT ACC ACC TAT Jδ1 CCA TGG GAA CTC ATC TTT (SEQ ID NO:7)

e) Integration into the expression vector and transfection into eukaryotic cells

The digestion, with the XhoI and EcoRI restriction enzymes, of the vector pBS-SK containing the Vδ1 Cδ cDNA liberates a DNA fragment encoding the entire variable part Vδ1 Dδ2 Jδ1 and the portion of the first exon of the constant part Cδ between the joining region and the unique EcoRI site.

This DNA fragment was purified and integrated into the expression vector pKCR6 containing the soluble Vδ3 chain after it had been digested with the XhoI and EcoRI restriction enzymes. This strategy therefore made it possible to replace the variable part Vδ3 with the variable part Vδ1 and thus to construct a cDNA encoding a soluble Vδ1 chain.

The vector thus obtained was co-transfected in combination with the expression vector containing the cDNA encoding the soluble Vγ9 chain.

The procedure for transfection, screening of the producing clones and purification of the soluble TCRs produced is analogous to that described for the production of soluble TCR Vγ9 Vδ2.

## 2. Detection And Purification of Other Soluble Tγδ Receptors

a) Detection of various soluble receptors, control of specificity

In the same manner as described above, 2 IRMAs were developed with the antibody 510 as phase antibody and with the antibodies 360 and 389 as tracers. These 2 IRMAs were tested on the supernatants of CHOs transfected with the genes Vγ9/Vδ2, Vγ9/Vδ3, Vγ8/Vδ2. Only the tracers corresponding to the transfected V give a signal, thus providing a good control of specificity.

b) Development of a general method of purification

The purification described previously for isolating the Vγ9Vδ2 receptor consisted of an immunopurification with an anti-Vγ9 antibody (Y102 or 7B6). An affinity column of the same type but using the antibody 510 described above and which recognizes a determinant of the delta constant chain was used. The advantage of this new purification is the possibility of purifying any soluble receptor of the invention regardless of the γ, δ and even α, β variable chains which they contain. This method was first tested in order to purify the soluble receptor containing Vγ9/Vδ3.

5 mg of antibody 510 were covalently linked to 1 g of a matrix of cyanogen bromide-activated sepharose 4B beads (PHARMACIA, Upsalla, Sweden) according to the instructions of the supplier.

The supernatant from a culture of the transfectant γ9δ3 was applied to the affinity column thus formed at the rate of 10 ml/hour at room temperature. After washing with a phosphate buffered saline solution PBS (0.01M phosphate, 0.14M NaCl, pH 7.2, same flow rate), the bound material was eluted with a 0.05M titrate solution at pH 3.0. The eluted fractions were neutralized immediately with a 0.2M Tris buffer pH 9 (100 μl for 1 ml of eluate).

The fractions positive for the soluble TR activity as attested by the IRMA test were combined and concentrated to 1 μg/ml of proteins on a CENTRICON cell (30 KD barrier) (AMICON, Beverly, Mass., USA) according to the instructions of the manufacturer. This cell also made it possible to change the buffer for PBS.

The analysis of the eluted proteins was carried out by SDS-PAGE and by Western-blotting. The analysis gave

slightly different results compared with γ9δ2. Indeed, under non-reducing conditions, three highly predominant bands of molecular weights 65, 68, 70 kD, which separated into four predominant bands 32.5, 34, 36 and 40 kD [sic]. Western-blot analysis with the anti-bodies 510 (anti-Cδ) and 360 (anti-Vγ9) showed that all the predominant bands previously observed under non-reducing conditions reacted with both antibodies. Under reducing conditions, the bands reacted either with the antibody 360 or with the antibody 510.

From this analysis, it can be concluded that the material eluted from the affinity column consisted essentially of covalently linked γδ heterodimers possibly present in the form of several glycosylation isomers.

SEQUENCE LISTING

( 1 ) GENERAL INFORMATION:

( i i i ) NUMBER OF SEQUENCES: 19

( 2 ) INFORMATION FOR SEQ ID NO:1:

( i ) SEQUENCE CHARACTERISTICS:
( A ) LENGTH: 24 base pairs
( B ) TYPE: nucleic acid
( C ) STRANDEDNESS: single
( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: DNA (genomic)

( x ) PUBLICATION INFORMATION:
( H ) DOCUMENT NUMBER: WO 94/12648
( I ) FILING DATE: 25-NOV-1993
( J ) PUBLICATION DATE: 09-JUN- 1994

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GGGTTACTGC AGCAGTAGTG TATC 24

( 2 ) INFORMATION FOR SEQ ID NO:2:

( i ) SEQUENCE CHARACTERISTICS:
( A ) LENGTH: 27 base pairs
( B ) TYPE: nucleic acid
( C ) STRANDEDNESS: single
( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: DNA (genomic)

( x ) PUBLICATION INFORMATION:
( H ) DOCUMENT NUMBER: WO 94/12648
( I ) FILING DATE: 25-NOV-1993
( J ) PUBLICATION DATE: 09-JUN- 1994

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CCCTCGAGAT GCTGTTGGCT CTAGCTC 27

( 2 ) INFORMATION FOR SEQ ID NO:3:

( i ) SEQUENCE CHARACTERISTICS:
( A ) LENGTH: 39 base pairs
( B ) TYPE: nucleic acid
( C ) STRANDEDNESS: single
( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: DNA (genomic)

( x ) PUBLICATION INFORMATION:
( H ) DOCUMENT NUMBER: WO 94/12648
( I ) FILING DATE: 25-NOV-1993
( J ) PUBLICATION DATE: 09-JUN- 1994

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TGTGCCACCT GGGACAGTCA TTATTATAAG AAACTCTTT 39

( 2 ) INFORMATION FOR SEQ ID NO:4:

( i ) SEQUENCE CHARACTERISTICS:
( A ) LENGTH: 27 base pairs
( B ) TYPE: nucleic acid
( C ) STRANDEDNESS: single
( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: DNA (genomic)

( x ) PUBLICATION INFORMATION:
( H ) DOCUMENT NUMBER: WO 94/12648
( I ) FILING DATE: 25-NOV-1993
( J ) PUBLICATION DATE: 09-JUN- 1994

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GGGTTACTTC TCGGTATGAA CTATGGC 27

( 2 ) INFORMATION FOR SEQ ID NO:5:

( i ) SEQUENCE CHARACTERISTICS:
( A ) LENGTH: 30 base pairs
( B ) TYPE: nucleic acid
( C ) STRANDEDNESS: single
( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: DNA (genomic)

( x ) PUBLICATION INFORMATION:
( H ) DOCUMENT NUMBER: WO 94/12648
( I ) FILING DATE: 25-NOV-1993
( J ) PUBLICATION DATE: 09-JUN- 1994

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GACTCGAGAA AAGATGATTC TTACTGTGGG 30

( 2 ) INFORMATION FOR SEQ ID NO:6:

( i ) SEQUENCE CHARACTERISTICS:
( A ) LENGTH: 42 base pairs
( B ) TYPE: nucleic acid
( C ) STRANDEDNESS: single
( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: DNA (genomic)

( x ) PUBLICATION INFORMATION:
( H ) DOCUMENT NUMBER: WO 94/12648
( I ) FILING DATE: 25-NOV-1993
( J ) PUBLICATION DATE: 09-JUN- 1994

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:6:

ACTTACTGTC CTTTTTCCCG GCTCTTGGGG GACACCGATA AA 42

( 2 ) INFORMATION FOR SEQ ID NO:7:

( i ) SEQUENCE CHARACTERISTICS:
( A ) LENGTH: 60 base pairs
( B ) TYPE: nucleic acid
( C ) STRANDEDNESS: single
( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: DNA (genomic)

( x ) PUBLICATION INFORMATION:
( H ) DOCUMENT NUMBER: WO 94/12648
( I ) FILING DATE: 25-NOV-1993
( J ) PUBLICATION DATE: 09-JUN- 1994

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:7:

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( 2 ) INFORMATION FOR SEQ ID NO:8:

( i ) SEQUENCE CHARACTERISTICS:
( A ) LENGTH: 26 base pairs
( B ) TYPE: nucleic acid
( C ) STRANDEDNESS: single
( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: DNA (genomic)

( x ) PUBLICATION INFORMATION:
( H ) DOCUMENT NUMBER: WO 94/12648
( I ) FILING DATE: 25-NOV-1993
( J ) PUBLICATION DATE: 09-JUN- 1994

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:8:

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( 2 ) INFORMATION FOR SEQ ID NO:9:

( i ) SEQUENCE CHARACTERISTICS:
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( B ) TYPE: nucleic acid
( C ) STRANDEDNESS: single
( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: DNA (genomic)

( i x ) FEATURE:
( A ) NAME/KEY: CDS
( B ) LOCATION: 1..48

( x ) PUBLICATION INFORMATION:
( H ) DOCUMENT NUMBER: WO 94/12648
( I ) FILING DATE: 25-NOV-1993
( J ) PUBLICATION DATE: 09-JUN- 1994

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:9:

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Ser Cys His Lys Pro Lys Ala Ile Val His Thr Glu Lys Val Asn Met
 1               5                  10                  15
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( 2 ) INFORMATION FOR SEQ ID NO:10:

( i ) SEQUENCE CHARACTERISTICS:
( A ) LENGTH: 16 amino acids
( B ) TYPE: amino acid
( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: protein

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:10:

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Ser Cys His Lys Pro Lys Ala Ile Val His Thr Glu Lys Val Asn Met
 1               5                  10                  15
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( 2 ) INFORMATION FOR SEQ ID NO:11:

( i ) SEQUENCE CHARACTERISTICS:
( A ) LENGTH: 27 base pairs
( B ) TYPE: nucleic acid
( C ) STRANDEDNESS: single
( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: DNA (genomic)

( x ) PUBLICATION INFORMATION:
( H ) DOCUMENT NUMBER: WO 94/12648
( I ) FILING DATE: 25-NOV-1993
( J ) PUBLICATION DATE: 09-JUN- 1994

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CGGTATCAAG TATGGCTCTT CATTGGG 27

( 2 ) INFORMATION FOR SEQ ID NO:12:

-continued

( i ) SEQUENCE CHARACTERISTICS:
( A ) LENGTH: 26 base pairs
( B ) TYPE: nucleic acid
( C ) STRANDEDNESS: single
( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: DNA (genomic)

( x ) PUBLICATION INFORMATION:
( H ) DOCUMENT NUMBER: WO 94/12648
( I ) FILING DATE: 25-NOV-1993
( J ) PUBLICATION DATE: 09-JUN- 1994

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:12:

AGCCCGGGCA GACATGCTGT CACTGC 26

( 2 ) INFORMATION FOR SEQ ID NO:13:

( i ) SEQUENCE CHARACTERISTICS:
( A ) LENGTH: 48 base pairs
( B ) TYPE: nucleic acid
( C ) STRANDEDNESS: single
( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: DNA (genomic)

( i x ) FEATURE:
( A ) NAME/KEY: CDS
( B ) LOCATION: 1..48

( x ) PUBLICATION INFORMATION:
( H ) DOCUMENT NUMBER: WO 94/12648
( I ) FILING DATE: 25-NOV-1993
( J ) PUBLICATION DATE: 09-JUN- 1994

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:13:

AAT TGT TCA AAA GAT GCA AAT GAT ACA CTA CTG CTG CAG CTC ACA AAC 48
Asn Cys Ser Lys Asp Ala Asn Asp Thr Leu Leu Leu Gln Leu Thr Asn
1 5 10 15

( 2 ) INFORMATION FOR SEQ ID NO:14:

( i ) SEQUENCE CHARACTERISTICS:
( A ) LENGTH: 16 amino acids
( B ) TYPE: amino acid
( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: protein

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Asn Cys Ser Lys Asp Ala Asn Asp Thr Leu Leu Leu Gln Leu Thr Asn
1 5 10 15

( 2 ) INFORMATION FOR SEQ ID NO:15:

( i ) SEQUENCE CHARACTERISTICS:
( A ) LENGTH: 24 base pairs
( B ) TYPE: nucleic acid
( C ) STRANDEDNESS: single
( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: DNA (genomic)

( x ) PUBLICATION INFORMATION:
( H ) DOCUMENT NUMBER: WO 94/12648
( I ) FILING DATE: 25-NOV-1993
( J ) PUBLICATION DATE: 09-JUN- 1994

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:15:

CTATGTGATG ACGACGTCAT TGGG 24

( 2 ) INFORMATION FOR SEQ ID NO:16:

( i ) SEQUENCE CHARACTERISTICS:
( A ) LENGTH: 783 base pairs
( B ) TYPE: nucleic acid
( C ) STRANDEDNESS: single
( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: DNA (genomic)

( i x ) FEATURE:
( A ) NAME/KEY: CDS
( B ) LOCATION: 1..783

( x ) PUBLICATION INFORMATION:
( H ) DOCUMENT NUMBER: WO 94/12648
( I ) FILING DATE: 25-NOV-1993
( J ) PUBLICATION DATE: 09-JUN- 1994

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:16:

```
ATG CAG AGG ATC TCC TCC CTC ATC CAT CTC TCT CTC TTC TGG GCA GGA    48
Met Gln Arg Ile Ser Ser Leu Ile His Leu Ser Leu Phe Trp Ala Gly
  1               5                  10                  15

GTC ATG TCA GCC ATT GAG TTG GTG CCT GAA CAC CAA ACA GTG CCT GTG    96
Val Met Ser Ala Ile Glu Leu Val Pro Glu His Gln Thr Val Pro Val
             20                  25                  30

TCA ATA GGG GTC CCT GCC ACC CTC AGG TGC TCC ATG AAA GGA GAA GCG   144
Ser Ile Gly Val Pro Ala Thr Leu Arg Cys Ser Met Lys Gly Glu Ala
         35                  40                  45

ATC GGT AAC TAC TAT ATC AAC TGG TAC AGG AAG ACC CAA GGT AAC ACA   192
Ile Gly Asn Tyr Tyr Ile Asn Trp Tyr Arg Lys Thr Gln Gly Asn Thr
     50                  55                  60

ATG ACT TTC ATA TAC CGA GAA AAG GAC ATC TAT GGC CCT GGT TTC AAA   240
Met Thr Phe Ile Tyr Arg Glu Lys Asp Ile Tyr Gly Pro Gly Phe Lys
 65                  70                  75                  80

GAC AAT TTC CAA GGT GAC ATT GAT ATT GCA AAG AAC CTG GCT GTA CTT   288
Asp Asn Phe Gln Gly Asp Ile Asp Ile Ala Lys Asn Leu Ala Val Leu
                 85                  90                  95

AAG ATA CTT GCA CCA TCA GAG AGA GAT GAA GGG TCT TAC TAC TGT GCC   336
Lys Ile Leu Ala Pro Ser Glu Arg Asp Glu Gly Ser Tyr Tyr Cys Ala
            100                 105                 110

TGT GAC ACC TTG GGG ATG GGG GGG GAA TAC ACC GAT AAA CTC ATC TTT   384
Cys Asp Thr Leu Gly Met Gly Gly Glu Tyr Thr Asp Lys Leu Ile Phe
        115                 120                 125

GGA AAA GGA ACC CGT GTG ACT GTG GAA CCA AGA AGT CAG CCT CAT ACC   432
Gly Lys Gly Thr Arg Val Thr Val Glu Pro Arg Ser Gln Pro His Thr
    130                 135                 140

AAA CCA TCC GTT TTT GTC ATG AAA AAT GGA ACA AAT GTC GCT TGT CTG   480
Lys Pro Ser Val Phe Val Met Lys Asn Gly Thr Asn Val Ala Cys Leu
145                 150                 155                 160

GTG AAG GAA TTC TAC CCC AAG GAT ATA AGA ATA AAT CTC GTG TCA TCC   528
Val Lys Glu Phe Tyr Pro Lys Asp Ile Arg Ile Asn Leu Val Ser Ser
                165                 170                 175

AAG AAG ATA ACA GAG TTT GAT CCT GCT ATT GTC ATC TCT CCC AGT GGG   576
Lys Lys Ile Thr Glu Phe Asp Pro Ala Ile Val Ile Ser Pro Ser Gly
            180                 185                 190

AAG TAC AAT GCT GTC AAG CTT GGT AAA TAT GAA GAT TCA AAT TCA GTG   624
Lys Tyr Asn Ala Val Lys Leu Gly Lys Tyr Glu Asp Ser Asn Ser Val
        195                 200                 205

ACA TGT TCA GTT CAA CAC GAC AAT AAA ACT GTG CAC TCC ACT GAC TTT   672
Thr Cys Ser Val Gln His Asp Asn Lys Thr Val His Ser Thr Asp Phe
    210                 215                 220

GAA GTG AAG ACA GAT TCT ACA GAT CAC GTA AAA CCA AAG GAA ACT GAA   720
Glu Val Lys Thr Asp Ser Thr Asp His Val Lys Pro Lys Glu Thr Glu
225                 230                 235                 240
```

-continued

AAC ACA AAG CAA CCT TCA AAG AGC TGC CAT AAA CCC AAA GCC ATA GTT 768
Asn Thr Lys Gln Pro Ser Lys Ser Cys His Lys Pro Lys Ala Ile Val
245 250 255

CAT ACC GAG AAG TAA 783
His Thr Glu Lys
260

( 2 ) INFORMATION FOR SEQ ID NO:17:

( i ) SEQUENCE CHARACTERISTICS:
( A ) LENGTH: 260 amino acids
( B ) TYPE: amino acid
( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: protein

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Met Gln Arg Ile Ser Ser Leu Ile His Leu Ser Leu Phe Trp Ala Gly
1 5 10 15

Val Met Ser Ala Ile Glu Leu Val Pro Glu His Gln Thr Val Pro Val
20 25 30

Ser Ile Gly Val Pro Ala Thr Leu Arg Cys Ser Met Lys Gly Glu Ala
35 40 45

Ile Gly Asn Tyr Tyr Ile Asn Trp Tyr Arg Lys Thr Gln Gly Asn Thr
50 55 60

Met Thr Phe Ile Tyr Arg Glu Lys Asp Ile Tyr Gly Pro Gly Phe Lys
65 70 75 80

Asp Asn Phe Gln Gly Asp Ile Asp Ile Ala Lys Asn Leu Ala Val Leu
85 90 95

Lys Ile Leu Ala Pro Ser Glu Arg Asp Glu Gly Ser Tyr Tyr Cys Ala
100 105 110

Cys Asp Thr Leu Gly Met Gly Gly Glu Tyr Thr Asp Lys Leu Ile Phe
115 120 125

Gly Lys Gly Thr Arg Val Thr Val Glu Pro Arg Ser Gln Pro His Thr
130 135 140

Lys Pro Ser Val Phe Val Met Lys Asn Gly Thr Asn Val Ala Cys Leu
145 150 155 160

Val Lys Glu Phe Tyr Pro Lys Asp Ile Arg Ile Asn Leu Val Ser Ser
165 170 175

Lys Lys Ile Thr Glu Phe Asp Pro Ala Ile Val Ile Ser Pro Ser Gly
180 185 190

Lys Tyr Asn Ala Val Lys Leu Gly Lys Tyr Glu Asp Ser Asn Ser Val
195 200 205

Thr Cys Ser Val Gln His Asp Asn Lys Thr Val His Ser Thr Asp Phe
210 215 220

Glu Val Lys Thr Asp Ser Thr Asp His Val Lys Pro Lys Glu Thr Glu
225 230 235 240

Asn Thr Lys Gln Pro Ser Lys Ser Cys His Lys Pro Lys Ala Ile Val
245 250 255

His Thr Glu Lys
260

( 2 ) INFORMATION FOR SEQ ID NO:18:

( i ) SEQUENCE CHARACTERISTICS:
( A ) LENGTH: 825 base pairs
( B ) TYPE: nucleic acid
( C ) STRANDEDNESS: single
( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: DNA (genomic)

( i x ) FEATURE:
( A ) NAME/KEY: CDS
( B ) LOCATION: 1..825

( x ) PUBLICATION INFORMATION:
( H ) DOCUMENT NUMBER: WO 94/12648
( I ) FILING DATE: 25-NOV-1993
( J ) PUBLICATION DATE: 09-JUN- 1994

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:18:

```
ATG CTG TCA CTG CTC CAC GCA TCA ACG CTG GCA GTC CTT GGG GCT CTG      48
Met Leu Ser Leu Leu His Ala Ser Thr Leu Ala Val Leu Gly Ala Leu
 1               5                  10                  15

TGT GTA TAT GGT GCA GGT CAC CTA GAG CAA CCT CAA ATT TCC AGT ACT      96
Cys Val Tyr Gly Ala Gly His Leu Glu Gln Pro Gln Ile Ser Ser Thr
            20                  25                  30

AAA ACG CTG TCA AAA ACA GCC CGC CTG GAA TGT GTG GTG TCT GGA ATA     144
Lys Thr Leu Ser Lys Thr Ala Arg Leu Glu Cys Val Val Ser Gly Ile
        35                  40                  45

ACA ATT TCT GCA ACA TCT GTA TAT TGG TAT CGA GAG AGA CCT GGT GAA     192
Thr Ile Ser Ala Thr Ser Val Tyr Trp Tyr Arg Glu Arg Pro Gly Glu
    50                  55                  60

GTC ATA CAG TTC CTG GTG TCC ATT TCA TAT GAC GGC ACT GTC AGA AAG     240
Val Ile Gln Phe Leu Val Ser Ile Ser Tyr Asp Gly Thr Val Arg Lys
65                  70                  75                  80

GAA TCC GGC ATT CCG TCA GGC AAA TTT GAG GTG GAT AGG ATA CCT GAA     288
Glu Ser Gly Ile Pro Ser Gly Lys Phe Glu Val Asp Arg Ile Pro Glu
                85                  90                  95

ACG TCT ACA TCC ACT CTC ACC ATT CAC AAT GTA GAG AAA CAG GAC ATA     336
Thr Ser Thr Ser Thr Leu Thr Ile His Asn Val Glu Lys Gln Asp Ile
            100                 105                 110

GCT ACC TAC TAC TGT GCC TTG TGG GAG GCC CAG CAA GAG TTG GGC AAA     384
Ala Thr Tyr Tyr Cys Ala Leu Trp Glu Ala Gln Gln Glu Leu Gly Lys
        115                 120                 125

AAA ATC AAG GTA TTT GGT CCC GGA ACA AAG CTT ATC ATT ACA GAT AAA     432
Lys Ile Lys Val Phe Gly Pro Gly Thr Lys Leu Ile Ile Thr Asp Lys
    130                 135                 140

CAA CTT GAT GCA GAT GTT TCC CCC AAG CCC ACT ATT TTT CTT CCT TCA     480
Gln Leu Asp Ala Asp Val Ser Pro Lys Pro Thr Ile Phe Leu Pro Ser
145                 150                 155                 160

ATT GCT GAA ACA AAG CTC CAG AAG GCT GGA ACA TAC CTT TGT CTT CTT     528
Ile Ala Glu Thr Lys Leu Gln Lys Ala Gly Thr Tyr Leu Cys Leu Leu
                165                 170                 175

GAG AAA TTT TTC CCT GAT GTT ATT AAG ATA CAT TGG GAA GAA AAG AAG     576
Glu Lys Phe Phe Pro Asp Val Ile Lys Ile His Trp Glu Glu Lys Lys
            180                 185                 190

AGC AAC ACG ATT CTG GGA TCC CAG GAG GGG AAC ACC ATG AAG ACT AAT     624
Ser Asn Thr Ile Leu Gly Ser Gln Glu Gly Asn Thr Met Lys Thr Asn
        195                 200                 205

GAC ACA TAC ATG AAA TTT AGC TGG TTA ACG GTG CCA GAA AAG TCA CTG     672
Asp Thr Tyr Met Lys Phe Ser Trp Leu Thr Val Pro Glu Lys Ser Leu
    210                 215                 220

GAC AAA GAA CAC AGA TGT ATC GTC AGA CAT GAG AAT AAT AAA AAC GGA     720
Asp Lys Glu His Arg Cys Ile Val Arg His Glu Asn Asn Lys Asn Gly
225                 230                 235                 240

GTT GAT CAA GAA ATT ATC TTT CCT CCA ATA AAG ACA GAT GTC ATC ACA     768
Val Asp Gln Glu Ile Ile Phe Pro Pro Ile Lys Thr Asp Val Ile Thr
                245                 250                 255

ATG GAT CCC AAA GAC AAT TGT TCA AAA GAT GCA AAT GAT ACA CTA CTG     816
Met Asp Pro Lys Asp Asn Cys Ser Lys Asp Ala Asn Asp Thr Leu Leu
```

-continued

| | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 260 | | | | | 265 | | | | | 270 | | | | | | |
| CTG | CAG | TAA | | | | | | | | | | | | | | | | | 825 |
| Leu | Gln | | | | | | | | | | | | | | | | | | |
| | | 275 | | | | | | | | | | | | | | | | | |

( 2 ) INFORMATION FOR SEQ ID NO:19:

( i ) SEQUENCE CHARACTERISTICS:
( A ) LENGTH: 274 amino acids
( B ) TYPE: amino acid
( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: protein

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:19:

```
Met Leu Ser Leu Leu His Ala Ser Thr Leu Ala Val Leu Gly Ala Leu
  1               5                  10                  15

Cys Val Tyr Gly Ala Gly His Leu Glu Gln Pro Gln Ile Ser Ser Thr
             20                  25                  30

Lys Thr Leu Ser Lys Thr Ala Arg Leu Glu Cys Val Val Ser Gly Ile
         35                  40                  45

Thr Ile Ser Ala Thr Ser Val Tyr Trp Tyr Arg Glu Arg Pro Gly Glu
     50                  55                  60

Val Ile Gln Phe Leu Val Ser Ile Ser Tyr Asp Gly Thr Val Arg Lys
 65                  70                  75                  80

Glu Ser Gly Ile Pro Ser Gly Lys Phe Glu Val Asp Arg Ile Pro Glu
                 85                  90                  95

Thr Ser Thr Ser Thr Leu Thr Ile His Asn Val Glu Lys Gln Asp Ile
            100                 105                 110

Ala Thr Tyr Tyr Cys Ala Leu Trp Glu Ala Gln Gln Glu Leu Gly Lys
        115                 120                 125

Lys Ile Lys Val Phe Gly Pro Gly Thr Lys Leu Ile Ile Thr Asp Lys
    130                 135                 140

Gln Leu Asp Ala Asp Val Ser Pro Lys Pro Thr Ile Phe Leu Pro Ser
145                 150                 155                 160

Ile Ala Glu Thr Lys Leu Gln Lys Ala Gly Thr Tyr Leu Cys Leu Leu
                165                 170                 175

Glu Lys Phe Phe Pro Asp Val Ile Lys Ile His Trp Glu Glu Lys Lys
            180                 185                 190

Ser Asn Thr Ile Leu Gly Ser Gln Glu Gly Asn Thr Met Lys Thr Asn
        195                 200                 205

Asp Thr Tyr Met Lys Phe Ser Trp Leu Thr Val Pro Glu Lys Ser Leu
    210                 215                 220

Asp Lys Glu His Arg Cys Ile Val Arg His Glu Asn Asn Lys Asn Gly
225                 230                 235                 240

Val Asp Gln Glu Ile Ile Phe Pro Pro Ile Lys Thr Asp Val Ile Thr
                245                 250                 255

Met Asp Pro Lys Asp Asn Cys Ser Lys Asp Ala Asn Asp Thr Leu Leu
            260                 265                 270

Leu Gln
```

I claim:

1. Process for producing soluble T receptors, comprising co-transfecting into a host cell DNA sequences each encoding only a single peptide consisting of one of the constituent T receptor units, from which the transmembrane portion of the T receptor has been deleted.

2. Process according to claim 1, wherein VαC/60 /VβCβ soluble T receptors are produced by co-transfecting, into a host cell, said DNA sequences each encoding a respective one of the α and β subunits of the Tαβ receptor from which the transmembrane portion of the Tαβ receptor has been deleted.

3. Process according to claim 1, wherein $V\gamma C\gamma/V\delta C\delta$ soluble T receptors are produced by co-transfecting, into a host cell, said DNA sequences each encoding a respective one of the $\gamma$ and $\delta$ subunits of the $T\gamma\delta$ receptor from which the transmembrane portion of the $T\gamma\delta$ receptor has been deleted.

4. Process according to claim 1, wherein $V\alpha C/65$ /$V\beta C\delta$ heterodimeric soluble T receptors are produced, in which the constituent subunits are associated via a covalent bond, by co-transfecting, into a host cell, said DNA sequences each encoding a respective one of the $C\gamma$ and $C\delta$ domains of the $\gamma$ and $\delta$ subunits of the $T\gamma\delta$ receptor from which their transmembrane portion has been deleted, fused respectively to the DNA sequences encoding the $V\alpha$ and $V\beta$ domains of the $\alpha$ and $\beta$ subunits of the $T\alpha\beta$ receptor.

5. Process according to claim 1, wherein $V\alpha C\delta/V\beta C/65$ heterodimeric soluble T receptors are produced, in which the constituent subunits are associated via a covalent bond, by co-transfecting, into a host cell, said DNA sequences each encoding a respective one of the $C\gamma$ and $C\delta$ domains of the $\gamma$ and $\delta$ subunits of the $T\gamma\delta$ receptor from which their transmembrane portion has been deleted, fused respectively to the DNA sequences encoding the $V\beta$ and $V\alpha$ domains of the $\beta$ and $\alpha$ subunits of the $t\alpha\beta$ receptor.

6. Process according to claim 1, wherein $V\gamma C\gamma/V\alpha C\delta$ hybrid soluble T receptors are produced by co-transfecting, into a host cell, a said DNA sequence encoding the $\gamma$ subunit of the $T\gamma\delta$ receptor from which its transmembrane portion has been deleted, and a said DNA sequence encoding the $C\delta$ domain of the $\delta$ subunit fused to a said DNA sequence encoding the $V\alpha$ domain of the $\alpha$ subunit of the $T\alpha\beta$ receptor.

7. Process according to claim 1, wherein the deletion of the transmembrane portion of the constituent T receptor subunits is carried out by introducing a translational termination codon upstream of the sequences encoding the transmembrane portion of these subunits.

8. Process according to claim 7, wherein the introduction of a translational termination codon is effected by PCR directed mutagenesis.

9. Process according to claim 1, wherein the co-transfection is carried out into eukaryotic cells.

10. Process according to claim 9, wherein said eukaryotic cells are hamster ovary cells.

* * * * *

# ATTACHMENT 8

US005583031A

# United States Patent [19]

**Stern**

[11] **Patent Number: 5,583,031**

[45] **Date of Patent: Dec. 10, 1996**

[54] **EMPTY MAJOR HISTOCOMPATIBILITY CLASS II HETERODIMERS**

[75] Inventor: **Lawrence J. Stern**, Arlington, Mass.

[73] Assignee: **President and Fellows of Harvard College**, Cambridge, Mass.

[21] Appl. No.: **831,895**

[22] Filed: **Feb. 6, 1992**

[51] **Int. Cl.**[6] .............................. **C12N 5/06**; C12N 5/10; C07K 14/74

[52] **U.S. Cl.** .................. **435/240.2**; 435/69.3; 435/320.1; 530/395; 530/403; 530/868; 424/184.1; 424/185.1; 424/193.1; 514/8

[58] **Field of Search** .................................. 424/88, 184.1, 424/278.1, 185.1, 193.1; 514/2, 8; 530/395, 402, 403, 868; 435/320.1, 252.3, 69.1, 69.3, 70.3, 69.6, 240.2

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(List continued on next page.)

*Primary Examiner*—Kay K. A. Kim
*Assistant Examiner*—Thomas Cunningham
*Attorney, Agent, or Firm*—Fish & Richardson P.C.

[57] **ABSTRACT**

The invention features an isolated sample of mammalian class II major histocompatibility heterodimers which are membrane-associated or in soluble form, and which are capable of binding added antigenic peptide; methods for producing large amounts of the soluble or membrane-associated histocompatibility protein by expression of DNA encoding the $\alpha$ and $\beta$ polypeptides; and methods for loading these heterodimers with any desired antigen.

**18 Claims, 6 Drawing Sheets**

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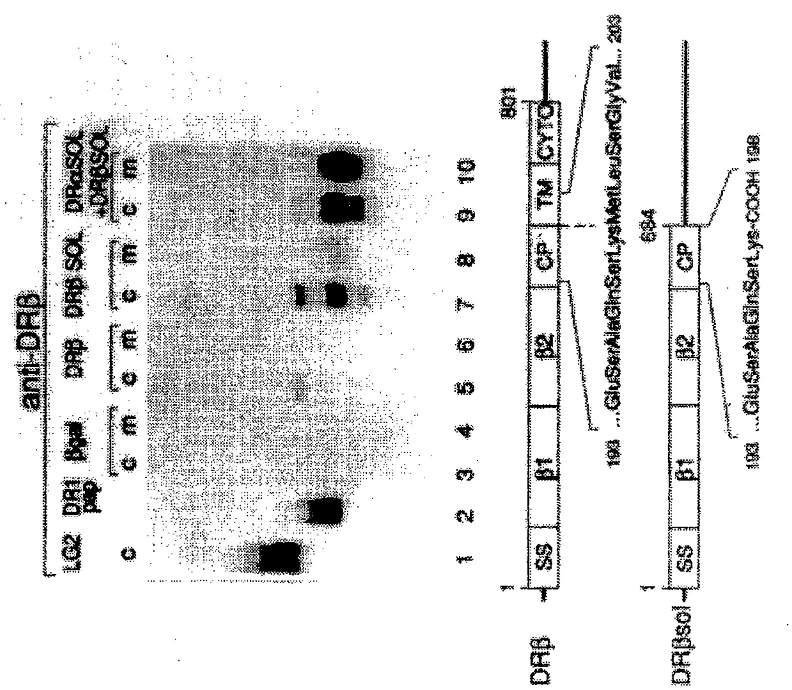
anti-DRβ
LG2
DR1 pep
βgal
DRβ
DRβ SOL
DRαSOL +DRβSOL
c m
1 2 3 4 5 6 7 8 9 10
DRβ
DRβsol
SS
β1
β2
CP
TM
CYTO
801
654
193 ...GluSerAlaGlnSerLysMetLeuSerGlyVal... 203
193 ...GluSerAlaGlnSerLys-COOH 198

FIG. 1B

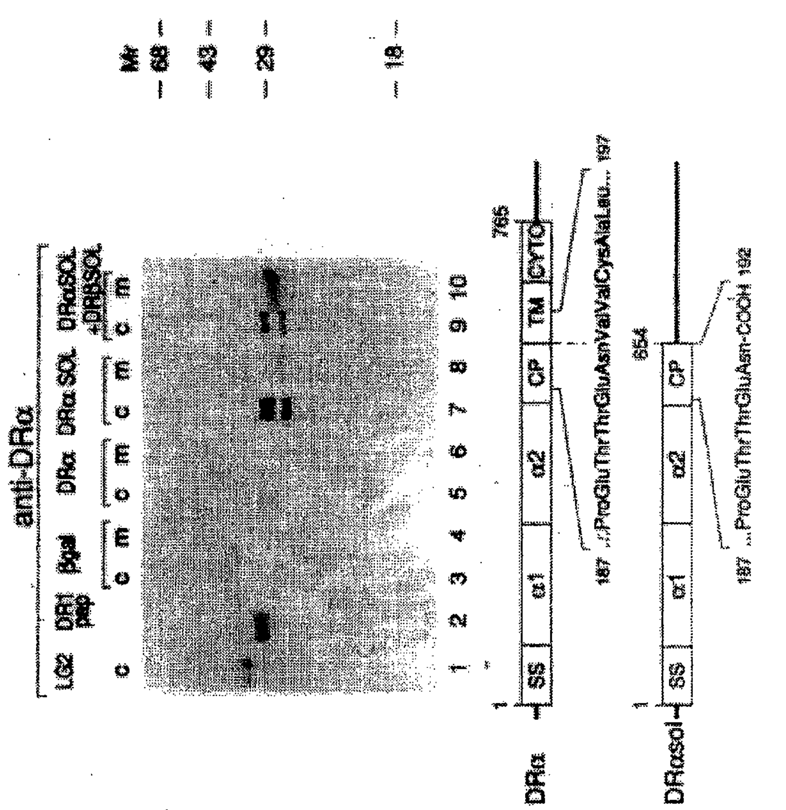
Mr
68
43
29
18
anti-DRα
LG2
DR1 pep
βgal
DRα
DRα SOL
DRαSOL +DRβSOL
c m
1 2 3 4 5 6 7 8 9 10
DRα
DRαsol
SS
α1
α2
CP
TM
CYTO
765
654
187 ...ProGluThrThrGluAsnValValCysAlaLeu... 197
187 ...ProGluThrThrGluAsn-COOH 192

FIG. 1A

FIG. 2A

FIG. 2B

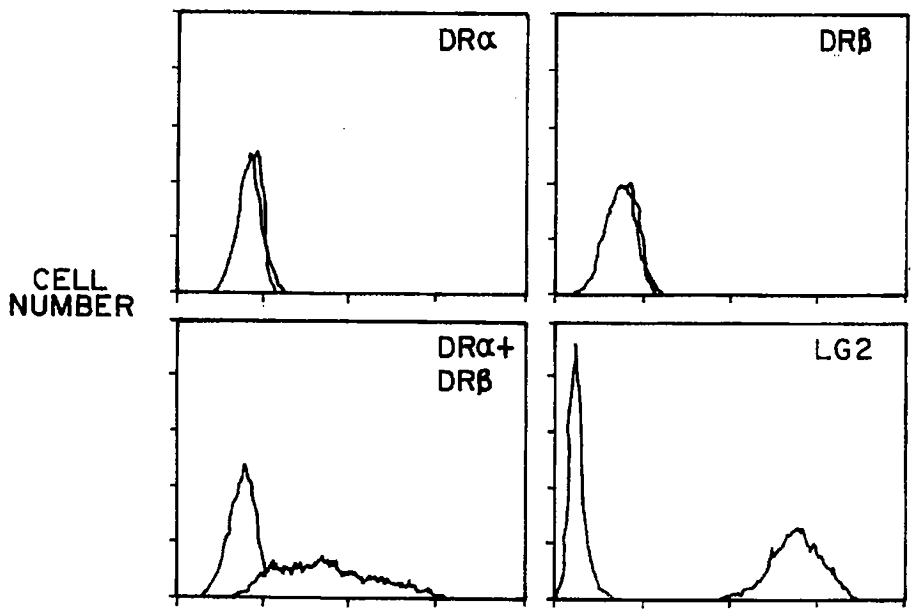


FIG. 2C

FIG. 2D

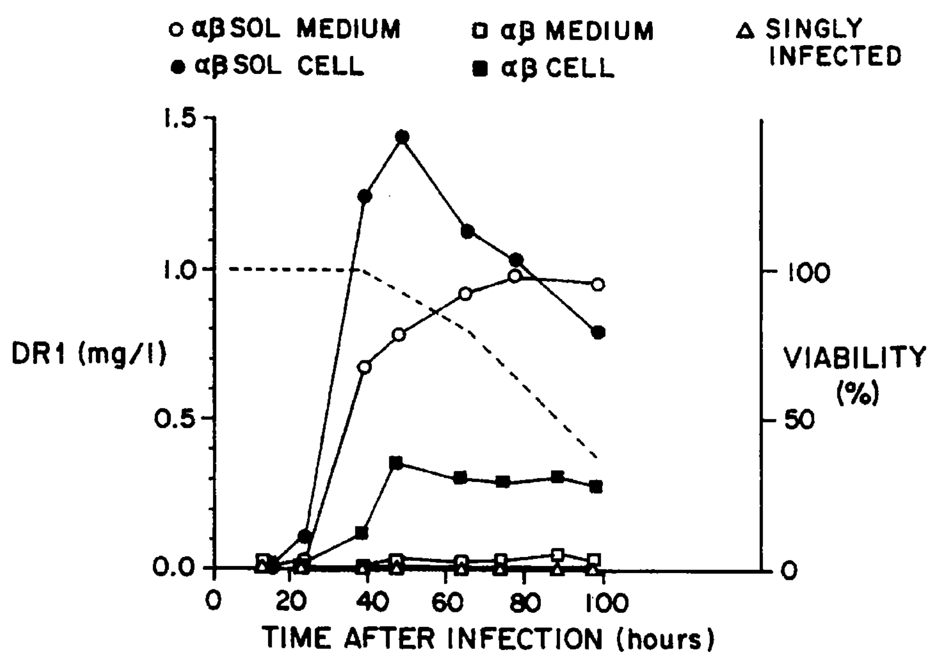


FIG. 3

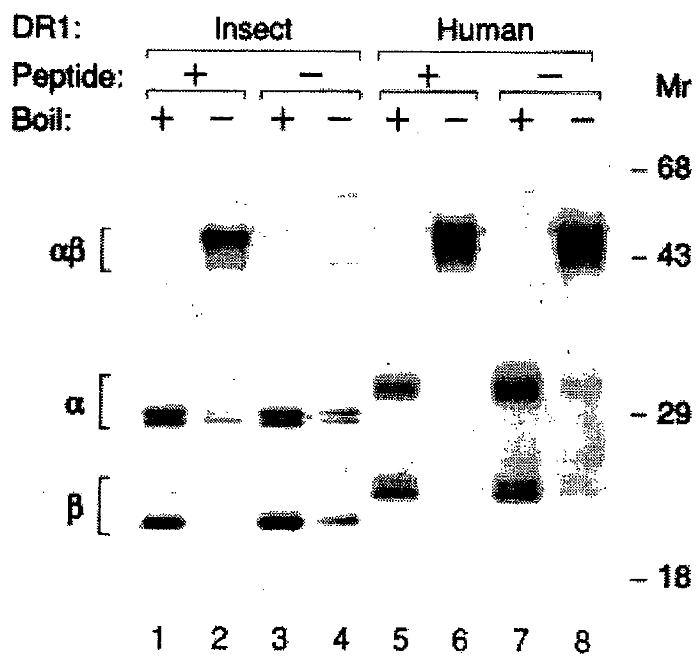


FIG. 4

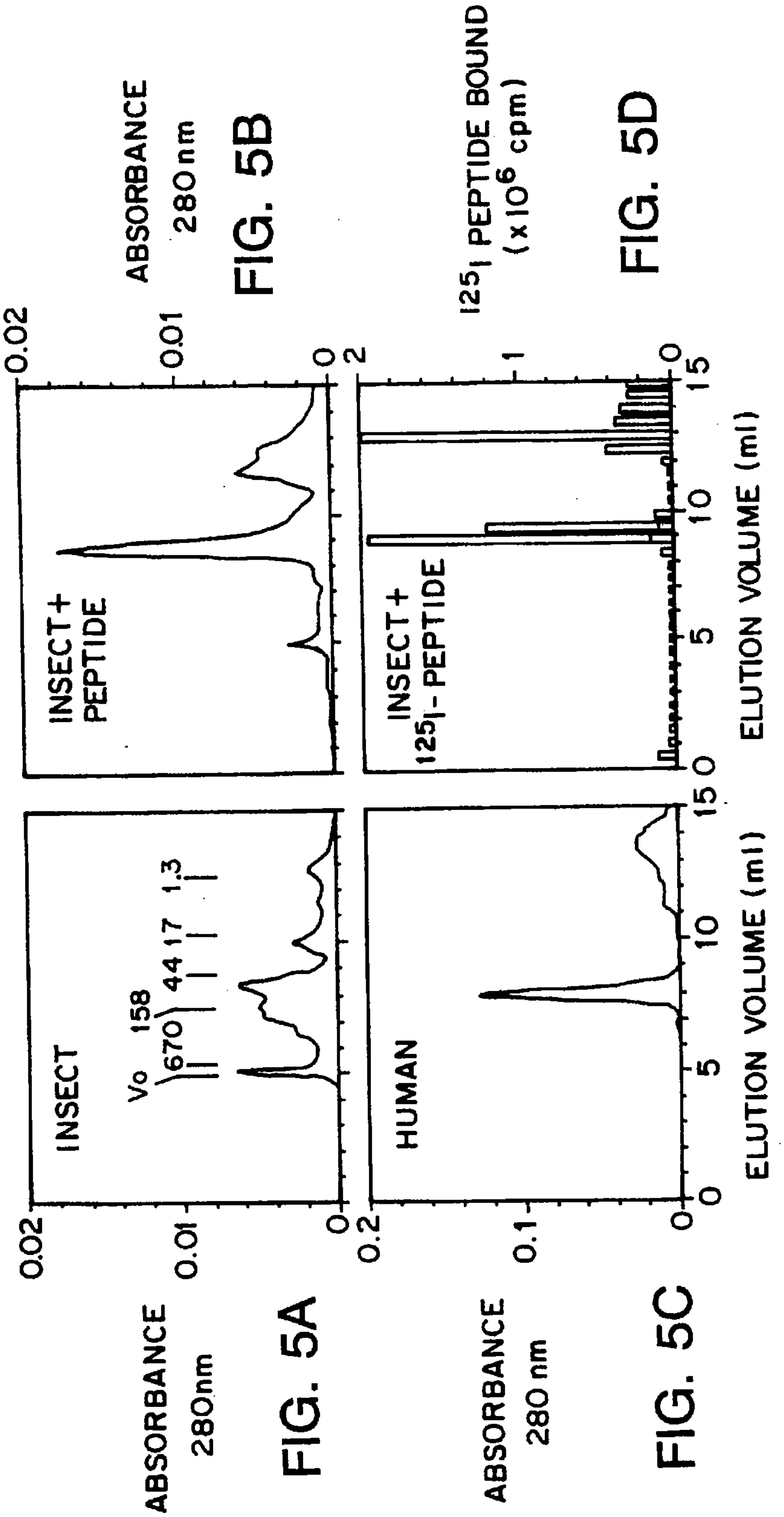


FIG. 5A

FIG. 5B

FIG. 5C

FIG. 5D

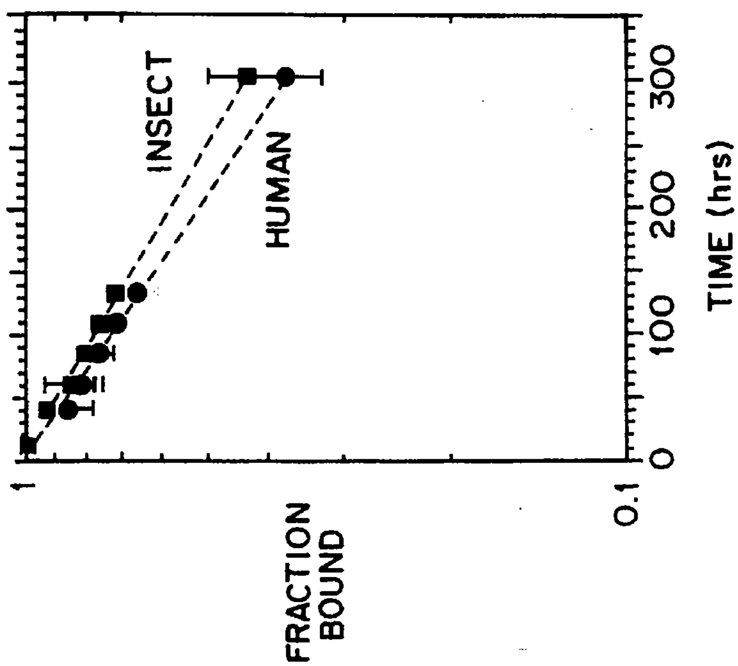
INSECT
HUMAN
FRACTION BOUND
1
0.1
0
100
200
300
TIME (hrs)

FIG. 6B

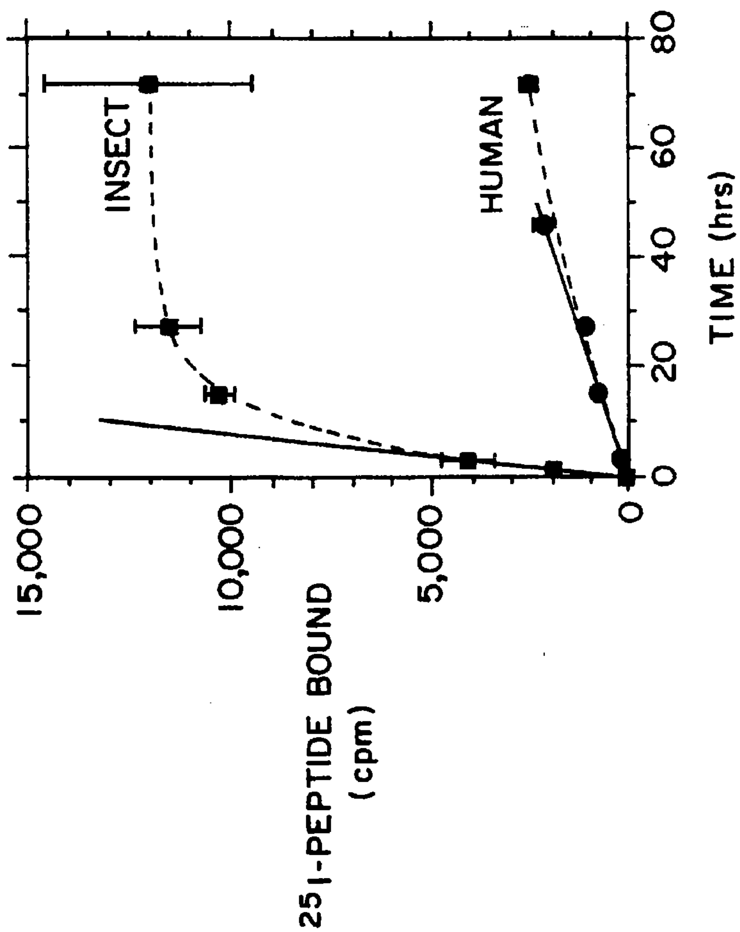
INSECT
HUMAN
125I-PEPTIDE BOUND (cpm)
15,000
10,000
5,000
0
0
20
40
60
80
TIME (hrs)

FIG. 6A

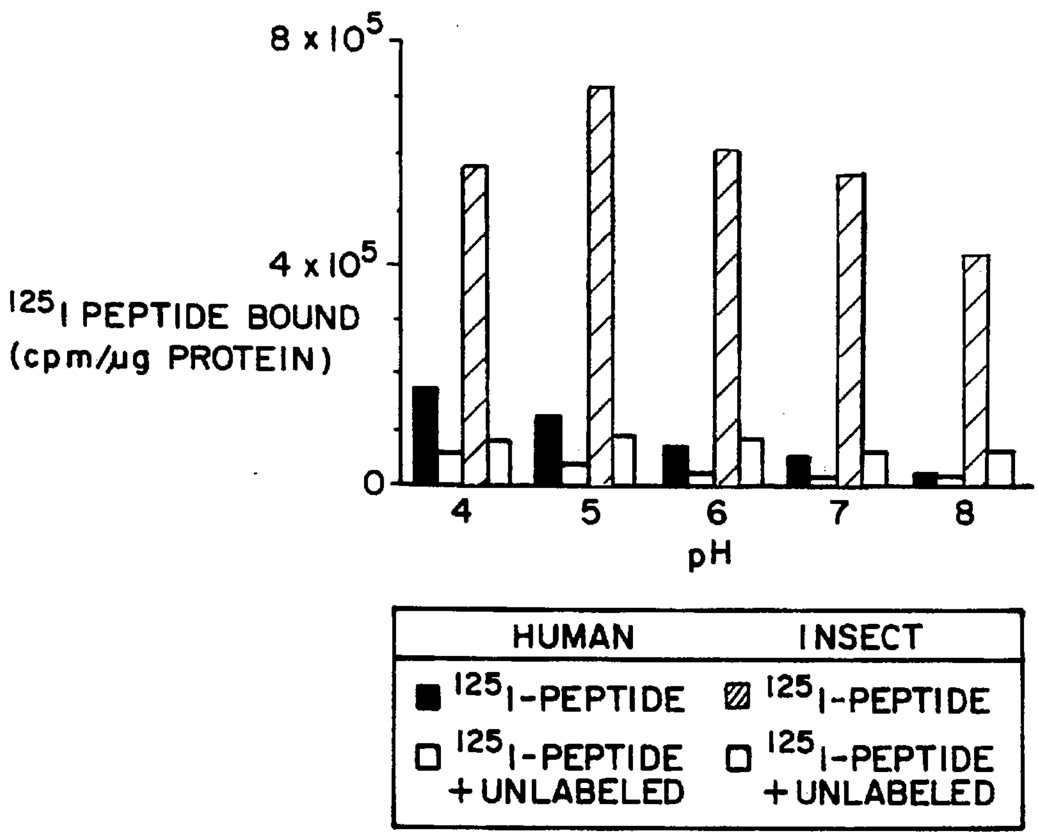
8 x 10^5
4 x 10^5
0
125I PEPTIDE BOUND
(cpm/µg PROTEIN)
4
5
6
7
8
pH
HUMAN
INSECT
125I-PEPTIDE
125I-PEPTIDE
125I-PEPTIDE
+UNLABELED
125I-PEPTIDE
+UNLABELED

FIG. 7

# EMPTY MAJOR HISTOCOMPATIBILITY CLASS II HETERODIMERS

## BACKGROUND OF THE INVENTION

The field of the invention is the major histocompatibility complex class II antigens and immune disorders.

Autoimmunity implies that an immune response has been generated against self-antigens (autoantigens). Central to the concept of autoimmunity is the breakdown in the ability of the immune system to differentiate between self- and non-self antigens. An abnormal immune response to self-antigens implies that there is a loss of tolerance.

The major histocompatibility complex (MHC) class II molecules are important for interactions between immune cells, particularly in antigen presentation to T cells. During a normal immune response, MHC molecules present a foreign antigen to a T cell as a non-self antigen. T cells respond by initiating a cascade of immune events that results in the eventual elimination of the foreign molecule. During autoimmune disease, MHC molecules present a self-antigen to the T cells as a non-self antigen, an event that also triggers T cell induced immune activation. However, in this latter case, since the immune response is directed against self-antigens it frequently results in severe damage to tissues and organs.

MHC proteins are highly polymorphic cell surface glycoproteins that bind antigenic peptides and display them at the cell surface (Rothbard and Gefter, 1991, Ann. Rev. Immunol. 9: 527). T lymphocytes initiate immune responses by recognizing a specific peptide bound to an MHC protein. Class I MHC proteins bind to endogenous peptides in the endoplasmic reticulum (Nuchtern et al., 1989, Nature 339: 223; Yewdell and Bennick, 1990, Cell 62: 203), while class II MHC proteins generally bind exogenously derived peptides in a specialized post-Golgi compartment (Guagliardi et al., 1990, Nature 343: 133; Neefjes et al., 1990, Cell 61: 171; Harding et al., 1990, Proc. Natl. Acad. Sci. USA 87: 5553; Davidson et al., 1991, Cell 67: 105; Germain and Hendrix, 1991, Nature 353: 134). Both class I and class II MHC proteins must bind peptides tightly to prevent peptide exchange at the cell surface and inappropriate immune response.

The peptide-binding sites of class I molecules are usually occupied with a mixture of peptides (Bjorkman et al. 1987, Nature 329: 506; Jardetzky et al., 1991, Nature 353: 326; Falk et al., 1991, Nature 351: 290), and class I molecules do not easily exchange or bind peptides in vitro (Chen and Parham, 1989, Nature 337: 743). Studies using mutant cell lines that do not load peptides onto class I molecules have suggested that peptide binding is required for assembly of the class I heterodimer and for stable cell surface expression (Townsend et al., 1989, Nature 340: 443; Townsend et al., 1990, Cell 62: 285; Ljunggren et al., 1990, Nature 346: 476; Ortiz-Navarrete and Hammerling, 1991, Proc. Natl. Acad. Sci. USA 88: 3594).

Class II MHC proteins isolated from lymphoid cells are very stable complexes with antigenic peptides (Buus et al., 1988, Science 242: 1045; Rudensky et al., 1991, Nature 353: 662). Less than 20% of these class II molecules will bind antigenic peptide added in vitro (Watts and McConnell, 1986, Proc. Natl. Acad. Sci. USA 83: 9660; Buus et al., 1987, Immunol. Rev. 98: 115; Jardetzky et al., 1990, Nature 353: 326; O'Sullivan et al., 1990, J. Immunol. 145: 1799; Roche and Cresswell, 1990, Ann. Rev. Immunol. 144: 1849), or in vivo (Ceppellini et al., 1989, Nature 339: 392;



Busch and Rotherbard, 1990, J. Immunol. Meth. 134: 1). The peptide-binding sites on the remainder of the proteins are occupied with tightly bound peptides (Tampe and McConnell, 1991, Proc. Natl. Acad. Sci. USA 88: 4661).

## SUMMARY OF THE INVENTION

The invention features compositions and methods for producing empty class II major histocompatibility heterodimers by expression in insect cell culture, and for loading these molecules with any desired antigen. The compositions and methods of the invention are superior to those previously available because they provide histocompatibility protein that can be 100% loaded with any peptide antigen, and because they provide a large amount of soluble or membrane-associated histocompatibility protein.

Accordingly, in one aspect, the invention features a pure sample of mammalian empty class II heterodimer containing an $\alpha$ and a $\beta$ polypeptide, which is either membrane-associated or in soluble form. When the heterodimer is membrane-associated, the $\alpha$ and $\beta$ polypeptides each contain the transmembrane domain that is normally present on the naturally occurring molecules. When the heterodimer is soluble, the transmembrane domain is absent from both the $\alpha$ and $\beta$ polypeptides.

By a "pure sample" is meant a heterodimer that does not have an antigen bound to it. The "antigen" to be loaded onto a heterodimer can be any substance with antigenic properties, for example, a protein or a peptide, a carbohydrate, a nucleic acid or a lipid, or any combination, fragment or combinations of fragments thereof. An "empty" heterodimer is one which does not have an antigen bound to it. A "membrane-associated" heterodimer is one which is complexed with a lipid membrane by virtue of an amino acid sequence which acts as a transmembrane domain, contained within each of the polypeptides comprising the heterodimer, and which anchors the heterodimer to a membrane. A "soluble" heterodimer is one which is not membrane-associated and wherein the polypeptides contained within the heterodimer do not contain an amino acid sequence acting as a transmembrane domain or as a cytoplasmic domain. An "antigenic peptide" is one which contains an amino acid sequence that encompasses an antigenic determinant. Such a peptide may be a full-length peptide which contains within it an antigenic determinant, or it may be a peptide whose amino acid sequence solely specifies an antigenic determinant. For the purposes of clarity, the term "antigenic peptide" will be used hereinafter to describe the molecule which can be bound to the empty heterodimer, although it is understood that this molecule need not be restricted solely to a peptide molecule.

The invention also features a baculovirus which contains DNA encoding the $\alpha$ polypeptide of the heterodimer and a baculovirus which contains DNA encoding the $\beta$ polypeptide of the heterodimer. In each case, the baculovirus contains DNA encoding either the membrane-associated or soluble form of each polypeptide.

In yet another aspect of the invention, there is described a method of producing either a membrane-associated or soluble empty major histocompatibility class II heterodimer. The method involves coinfecting insect cells with baculoviruses which contain DNA encoding the $\alpha$ and $\beta$ polypeptides. During virus replication in the cells, the genes encoding the polypeptides are expressed and the protein products are recovered from the cells or from their growth medium.

The invention also features a cell which expresses a membrane-associated or soluble major histocompatibility class II heterodimer.

Other features and advantages of the invention will be apparent from the following detailed description and from the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

The drawings will first be briefly described.

FIG. 1(A and B) depicts expression of full-length and truncated DRα and DRβ polypeptides in baculovirus-infected Sf9 insect cells. Upper panels: Western blots for DRα and DRβ. Sf9 cells were harvested 72 hour post-infection, and aliquots of cell lysate (c) and extracellular medium (m) were analyzed by 12.5% acrylamide SDS-PAGE and Western blotting with specific antisera directed against DRα (FIG. 1A) or DRβ (FIG. 1B). Lane 1, human LG2 cell lysate; lane 2, affinity-purified, papain-solubilized DR1 from LG2 cells (DR1pap); lanes 3 and 4, cell lysate and extracellular medium from Sf9 insect cells infected with control baculovirus BV-β-gal; lanes 5 and 6, cell lysate and extracellular medium from insect cells infected with either BV-DRα or BV-DRβ; lanes 7 and 8, cell lysate and extracellular medium from insect cells infected with either BV-DRβsol or BV-DRβsol; lanes 9 and 10, cell lysate and extracellular medium from insect cells coinfected with both BV-DRα and BV-DRβsol. Samples of lysates and extracellular medium represented $1\times10^5$ cells in (FIG. 1A) and $2.5\times10^4$ cells in (FIG. 1B). Papain solubilized DR1 from human cells (DR1pap) was used at 100 ng (FIG. 1A) and 25 ng (FIG. 1B) per lane.

FIG, 1(C and D): HLA-DR1 genes used to construct recombinant baculoviruses. Nucleotide numbering beginning at the initiation codon is indicated above the boxes. Amino acid numbering begins after the signal sequence at the N-terminus of the mature polypeptide. Portions of the amino acid sequence near the C-terminal end of the extracellular domain along with amino acid residue numbers are indicated below the boxes. DRα (FIG. 1C) and DRβ (FIG. 1D) contain the entire coding sequence of the parent cDNAs. DRαsol (FIG. 1C) and DRαsol (FIG. 1D) have been truncated just before the transmembrane domain as indicated. Open boxes indicate coding regions: SS, signal sequence; α1, α2 (FIG. 1C), β1, β2 (FIG. 1D), HLA extracellular domains; CP, connecting peptide; TM, transmembrane domain; CYTO, cytoplasmic domain.

FIG. 2(A–d) is graph of cell surface expression of DR1 in infected Sf9 cells. Baculovirus-infected Sf9 insect cells along with LG2 human lymphoblastoid cells were analyzed by flow cytometry at 48 hour post-infection. Surface expression of DR1 was detected using phycoerythrin-conjugated anti-DR1 monoclonal L243 (shaded). Background fluorescence was estimated with non-specific phycoerythrin-conjugated mouse antibody (open). (FIG. 2A) Sf9 cells infected with BV-DRα alone. (B) Sf9 cells infected with BV-DRβ alone. (FIG. 2C) Sf9 cells coinfected with BV-DRα+BV-DRβ. (FIG. 2D) LG2 cells.

FIG. 3 is a graph of the time course of expression of soluble and membrane-bound HLA-DR1 in insect cells. Sf9 cells ($10^6$ cells per ml) were coinfected with BV-DRα+BV-DRβ (squares) or with BV-DRαsol+BV-DRβsol (circles), or were singly infected with either BV-DRα or BV-DRβ alone (triangles). DR1 concentration in the extracellular medium (open symbols) or in cell lysates (closed symbols) was determined by ELISA, using the conformationally sensitive monoclonal antibody L243 as the capture antibody. Determinations with monoclonal antibody LB3.1 produced similar results. The dashed line indicates cell viability by trypan blue exclusion.

FIG. 4 is a gel depicting the analysis of soluble DR1 from insect and human cells. Soluble DR1 (80 μM) from insect cells (lanes 1–4) or papain-solubilized DR1 from human cells (lanes 5–8) was incubated in the presence (lanes 1, 2, 5 and 6) or absence (3, 4, 7, and 8) of 360 μM HA(306–318) peptide, for 100 hr at 37° C. After incubation, samples were mixed with SDS-PAGE loading buffer (final [SDS]=1%). One half of each sample was boiled for 3 min before loading (odd lanes); the other half was loaded without boiling (even lanes). Samples were analyzed by SDS-PAGE on 12.5% polyacrylamide with Coomassie brilliant blue R250 detection. Positions of molecular weight markers BSA (68000), ovalbumin (43000), carbonic anhydrase (29000), and β-lactoglobulin (18400) are indicated at right.

FIG. 5(A–D) is a graph of HPLC gel filtration analysis of soluble DR1 from insect and human cells. Soluble DR1 (80 μM) from insect cells was incubated in the absence (FIG. 5A) or the presence (FIG. 5B) of 500 μM HA(306–318) peptide for 86 hr at 37° C., before HPLC analysis. The elution profile of papain-solubilized DR1 from human cells (FIG. 5C) was unaltered by incubation with HA(306–318)peptide. In a separate experiment (FIG. 5D), 0.3 μM soluble DR1 from insect cells was incubated, with 1 μM [$^{125}$I]HA(306–318) peptide (open bars) or with labeled peptide and a 50-fold excess of unlabeled HA(306–318) peptide (shaded bars), and was analyzed by gel filtration HPLC. Fractions (0.5 ml) were collected, and the amount of radioactivity in each fraction was determined by gamma counting. The inset to (FIG. 1A) indicates the elution position of molecular weight standards blue dextran (void volume Vo), thyroglobulin (670,000), immunoglobulin G (158,000), ovalbumin (44,000), myoglobin (17,000), and vitamin B12 (1,300).

FIG. 6(A and B) is a graphical demonstration of the association and dissociation kinetics of antigenic peptide binding to HLA-DR1 from human and insect cells. FIG. 6A (left panel): association kinetics. Soluble DR1 isolated from insect cells (0.14 μM), or produced by papain cleavage of DR1 from human cells (0.21 μM), was incubated with 2.5 μM [$^{125}$I]HA(306–318) peptide at 37° C. At the indicated times the binding reaction was stopped and the amount of bound peptide was determined by immunoabsorption. Squares, soluble DR1 from coinfected insect cells: circles, papain-solubilized DR1 from human lymphoblastoid cells. Closed symbols, DR1+$^{125}$I-labeled HA peptide, open symbols, DR1+$^{125}$I-labeled HA peptide+20-fold excess cold HA peptide. Solid lines indicate the initial rate of peptide binding: dashed lines indicated the best fit single exponential equations, with τ=7.8 hr and a maximum of 12,000 cpm for insect-cell-produced DR1, and with τ=81 hr and an extrapolated maximum of 4,800 cpm for human-cell-produced DR1.

FIG. 6B (right panel): dissociation kinetics. DR1-peptide complexes were formed as described above, isolated by spin ultrafiltration, and diluted to 25 nM DR1 in binding buffer containing 0.25 mM unlabeled peptide. At the indicated times DR1-peptide complexes were again isolated and the amount of radiolabeled peptide remaining bound to DR1 was determined by gamma counting. Dashed lines indicated single exponential fits with τ=81 hr for DR1 from insect cells and τ=52 hr for DR1 from human cells.

FIG. 7 is a histogram depicting pH dependence of antigenic peptide binding to HLA-DR1 from human and insect cells. Soluble DR1 produced by insect cells (0.2 μM, shaded bars) or prepared by papain digestion of DR1 purified from human cells (0.35 μM, solid bars) was incubated with 1.8 μM [$^{125}$I]HA(306–318) peptide at 37° C., in 0.1M sodium citrate-phosphate buffer at the indicated pH. After 96 hr, the amount of radioactive peptide bound to DR1 was determined by spin ultrafiltration. Radiolabeled peptide binding

in the presence of 25 μM unlabeled peptide is indicated by open bars for DR1 from human cells and by lightly hatched bars for DR1 from insect cells.

## DETAILED DESCRIPTION OF THE INVENTION

### Expression of Heterodimers

Class II histocompatibility proteins are expressed as αβ heterodimers by insect cells (*Spodoptera frugiperda*, fall armyworm) infected with recombinant baculoviruses. The viruses carry genes coding for the α and for the β subunits of the histocompatibility protein. The protein can be produced in a membrane-associated form, or in a secreted, soluble form by alteration of the carboxy-terminus. Like the mammalian cells from which histocompatibility proteins are conventionally isolated, the insect cells glycosylate and correctly assemble the histocompatibility protein, but, unlike the mammalian cells, they do not load the binding site with tightly bound endogenous peptides. The proteins are isolated from insect cells as empty molecules by immunoaffinity and ion-exchange procedures. Antigenic peptide is loaded onto the purified molecule in vitro, and the 1:1 complex of peptide and histocompatibility protein is isolated. The compositions and methods of the invention are described in detail below.

### Materials and Methods

Oligonucleotides were synthesized with a Milligen model 3700 DNA synthesizer using β-cyanoethyl phosphoroamidite chemistry, and were purified by denaturing acrylamide gel electrophoresis and reverse-phase chromatography on Sep-pack (Millipore) cartridges. Baculovirus transfer plasmids pVL1393 and pAC360-βgal and the wild-type baculovirus ACMNPV-E2 are available from In Vitrogen. Restriction enzymes and DNA modifying enzymes were obtained from New England Biolabs, Boehringer Mannheim, US Biochemicals and Promega.

Hybridoma cells secreting anti-DR monoclonal antibody L243 (1 $gG_{2a}$) were obtained from the American Type Culture Collection (ATCC #HB55) and were maintained in Dulbeccos modified Eaglets medium (DMEM: Sigma) plus 10% fetal bovine serum (FBS). As an alternative to L243, LB3.1-secreting (1 $gG_{2b}$) cells were obtained from J. Strominger (Harvard University) and were maintained in RPMI 1640 (Sigma) plus 10% FBS. For antibody, production cells were grown with immunoglobulin G-free FBS (Gibco) in roller bottle culture or in serum-free medium WHC935 medium (Amicon) in a min-Flow Path bioreactor. Antibodies were purified from clarified tissue culture medium by ammonium sulfate fractionation followed by affinity chromatography on protein A-agarose (Repligen) or protein G-Sepharose Fast Flow (Pharmacia). Phycoerythrin-conjugated L243 and control mouse immunoglobulin G were obtained from Becton-Dickinson. Rabbit antiserum against papain-solubilized DR1 was produced by Hazelton. Anti-DR1 monoclonal antibodies IVA12(β1), TAL14 1(β1), Tu36(β2), Tu39(β1), Tu43(αβ), and SG171(β1) and biotinylated monoclonal antibodies DA2 (β1), DA6. 147 (β1), DA6.321 (α), TALS. 1 (β1) were obtained from D. Vignali (Harvard University). As an alternative to these antibodies, L227(β1) and L243 (ATCC) were also used. The specificity of each antibody for DR domains is indicated in parentheses. Rabbit antisera specific for the α and β chains of DR1 was provided by D. Vignali (The Netherlands Cancer Institute). Such antisera can be prepared by any artisan skilled in the art by inoculating rabbits with publicly available α and β chains. Goat anti-rabbit or anti-mouse secondary antibodies were obtained from Boehringer Mannheim (horseradish peroxidase-labeled) and Promega (alkaline phosphate-labeled). Streptavidin-alkaline phosphatase was from Biorad.

Immunoaffinity-purified DR1 isolated from the human lymphoblastoid cell line LG2, and soluble DR1 produced by limited papain digestion of immunoaffinity-purified DR1 from LG2, were generous gifts of J. Gorga and J. Strominger (Harvard University). Glycosidases, digoxygenin-labeled lectins, and detergents were from Boehringer Mannheim. HA(306–318) peptide ($NH_2$-PKYVKQNTLKLAT-COOH) SEQ. ID NO: 5 was synthesized with an ABI model 431 peptide synthesizer using Fmoc chemistry, and was purified by reverse-phase high-pressure liquid chromatography (HPLC) on ClsProPep (Vydac) in 0.1% trifluoroacetic acid using a 40%–60% acetonitrile gradient. The purified peptide was characterized by amino acid analysis (Harvard Microsequenceing Facility) and by mass spectrometry (Harvard Spectrometry Lab) and shown to be homogenous. Peptide concentration was determined by ultraviolet absorbance using $\epsilon_{280}=1800\ M^{-1}cm^{311}$.

### Construction of Transfer Plasmids Carrying DRα, DRβ, Truncated DRα, and Truncated DRβ Genes

cDNA clones for the α and β subunits of HLA-DR1 were DRA and DRB1*0101, GENBANK identifiers: Hummhdram.pr and Hummhldr1b.pr. Transfer plasmids carrying DRα and DRβ genes were constructed by isolation of the genes as BamHI fragments from the appropriate cDNA clones and insertion of these genes into the unique BamHI site of the baculovirus transfer plasmid pVL1393. In this vector the inserted genes are under transcription control of the strong late polyhedrin promoter. The initiation codon of the polyhedrin gene has been altered to ATT (Luckow and Summers, 1989, Virology 170: 31), so that translation is initiated at the first ATG in the inserted gene. Clones carrying DRα or DRβ inserts in the proper orientation were isolated, and the expected sequences were confirmed throughout the entire coding regions.

DRαsol (FIG. 1) was constructed by using a synthetic oligonucleotide duplex that codes for DRα sequence from the unique PstI site at nucleotide 566 to the Asn-192 codon ending at nucleotide 651, followed by the termination codon TAA, and NotI and KpnI cloning sites. The sequences of the constituent oligonucleotides were:

5'-GGGTGGAGCACTGGGGCTTGGATGAGCCTCTTCTCAAGCATTGGGAATTC
GATGCTCCAAGCCCTCTCCCAGAGACTACAGAGAACTAAGCGGCCGCGGTAC-3' (SEQ ID NO:1)

and

3'-ACGTCCCACCTCGTGACCCCGAACCTACTCGGAGAAGAGTTCGTAACCCTTAAG
CTACGAGGTTCGGGAGAGGGTCTCTGATGTCTCTTGATTCGCCGGCGC-5' (SEQ ID NO:2)

Altered sequences relative to the DRα gene are underlined; the first two substitutions are silent changes to introduce a unique EcoRI site. They synthetic duplex was inserted into pVL1393-DRα between the PstI site in the DRα gene and KpnI site downstream in the disabled polyhedrin gene. One clone carrying the insert was sequenced

through the altered region and shown to have the expected sequence.

DRβsol (FIG. 1) was constructed by polymerase chain reaction-mediated amplification of the DRβ gene. The "forward" oligonucleotide primer complementary to the coding strand 5'-GACTTGGATCCTATAAATATGGTGTGTCTGAAGCTCCCT-3' (SEQ ID. NO: 3) introduces a BamHI site upstream of the initiation ATG codon, and the reverse primer 5'-ACAGCTCTAGATTACTTGCTCTGTGCAGATTCAGA-3' (SEQ ID NO: 4) introduces a termination TAA codon starting at nucleotide 682 followed by an XbaI cloning site. Sequences not present in the DRβ gene are underlined. The truncated gene was amplified by 10 cycles of melting (94° C., 1 min) annealing (55° C., 1 min) and extension (72° C., 3 min). The reaction product was isolated, cut with BamHI and XbaI and inserted into the corresponding restriction sites of pUC19. One of three clones sequenced had no unexpected substitutions and the DRβsol gene was excised from this clone and inserted between the BamHI and XbaI sites of pVL1393.

Construction of Recombinant Baculovirus Clones

Recombinant baculoviruses BV-βgal, BV-DRα, and BV-DRβ were produced by homologous recombination following cotransfection of $2\times10^6$ cells with 5 μg of plasmid and 1 μg of viral (wild-type ACMNPV-E2) DNAs, as described (Summers and Smith, 1988, A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures:Texas Agricultural Experiment Station Bulletin No. 1555, College Station, Tex.). Recombination efficiencies varied from 0.1% to 1%. Viral clones were isolated by limiting dilution in 96-well tissue culture plates. Recombinant viruses were identified by dot-blot DNA hybridization of alkali-lysed cells (Summers and Smith, Supra) using a $^{32}$P-labeled probe carrying both DRe and DRβ sequences. Three or four rounds of dilution and screening were required to obtain single isolates free of wild-type virus. Recombinant baculoviruses BV-DRαsol and BV-DRβsol were similarly produced and isolated except that BV-βgal viral DNA was used instead of wild-type ACMNPV-E2. This simplified the identification of nonrecombinant viruses which were easily observed by including 5-bromo-4-chloro-3-indole-β-D-galactoside (0.2 mg/ml) in the culture medium.

Sf9 Growth and Infection

Spodoptera frugiperda (Sf9) were obtained from the American Type Culture Collection (ATCC#CRL1711) and were maintained at 27° C. in TNM-FH medium (Gibco) plus 10% FBS. Viral stocks were produced by infection at low multiplicity and were stored at 4° C. Viral titers were usually greater than $10^8$ plaque-forming units per ml. For protein production, cells were grown in spinner flasks (100 ml or larger) in serum-free media SF900 (Gibco) or Excell410 (JRH Scientific). Cells were infected at $1\times10^6$ cells per ml with a multiplicity of infection of 20 for each virus using the procedures described in Summers and Smith (Supra).

SDS-PAGE and Western Blotting

Cell lysates for SDS-PAGE analysis were prepared by mixing washed cells with 1/10 culture volume phosphate-buffered saline (PBS: 20 mM phosphate 130 mM NaCl [pH 7.2]) containing 1% CHAPS and a mixture of protease inhibitors (1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 mM iodoacetamide, 0.3 μM aprotinin, 1 μM pepstatin, 1 μM leupeptin). The mixture was stored at 4° C. for 1 hr. and nuclei and cell debris were removed by low-speed centrifugation. Samples of extracellular medium for SDS-PAGE were prepared by acetone or trichloroacetic acid precipitation. Samples for SDS-PAGE were mixed with SDS-PAGE sample buffer (Laemmli, 1970, Nature 227: 680) containing 1% SDS and 1000 mM dithiothreitol (DTT) (final concentrations) and boiled for 3 min before application to 12.5% acrylamide SDS-PAGE slab gels (7×7×0.075 cm). After electrophoresis gels were transferred to polyvinylidene fluoride (PVDF) membranes (Immobilon-P Millipore). Membranes were blocked in 3% bovine serum albumin (BSA) in PBS. DRα and DRβ polypeptides were detected using appropriate antisera followed by alkaline-phosphatase conjugated anti-rabbit serum and nitro-blue tetrazolium and bromochloroindole phosphate as described (Blake et al., 1984, Anal. Biochem. 136: 175).

Flow Cytometry

Baculovirus-infected cells were analyzed by flow cytometry at 48 hr postinfection, before significant virus-induced cell lysis. In order to avoid the strong green autofluorescence intrinsic to Sf9 insect cells, long-wavelength flurorphore R-phycoerythrin (PE) was used. At 48 hr postinfection, $10^6$ cells were pelleted, gently resuspended in 1/10 culture volume Grace's medium (Gibco), 2% FBS, 0.01% $NAN_3$, and incubated for 1 hr on ice with PE-conjugated L243 or PE-conjugated nonspecific control mouse immunoglobulin G. The cells were washed three times with Grace's medium and were finally resuspended at 1/10 the initial culture volume in PBS and fixed with 2% paraformaldehyde. Red fluorescence was measured with a Becton-Dickinson FACS-can flow cytometer.

Enzyme-Linked Immunosorbent Assay (ELISA)

The ELISA used to measure DR1 concentration was a sandwich type, with solid phase L243 or LB3.1 monoclonal antibodies used to capture native DR1, and rabbit anti-DR1 and alkaline-phosphatase-labeled goat anti-rabbit antibodies used to detect bound DR1. Ninety-six-well microtiter plates (Maxisorp, Nunc) were coated with 200 ng of purified L243 or LB3.1 monoclonal antibody in 100 mM sodium carbonate (pH 9.6) blocked with 3% BSA in PBS, and stored at 4° C. All subsequent incubations were for 30 min or 1 hr at room temperature using 0.1 ml per well and were followed by three washes with 0.05% Triton X-100 in PBS (PBST). Dilutions of samples and DR1 standards (0.1–100 ng per well) were prepared in PBST plus 0.3% BSA and applied to the plate. After binding, DR1 was detecting using rabbit anti-DR1 serum (1:50,000 in PBST plus 0.3% BSA) followed by horseradish peroxidase-coupled goat anti-rabbit antibody (15 μg/ml in PBST plus 0.3% BSA). The plate was developed with the peroxidase substrate 2.2'azino-di[3-ethyl]benzthiazoline sulfonate (ABTS, Boehringer Mannheim) in perborate-citrate-phosphate buffer. After 5–15 min, the reaction was stopped with 0.2% $NAN_3$, and the absorbance at 405 nm was measured. For quantitation of DR1, triplicate sample dilutions were compared to a standard curve produced using purified, papain-solubilized DR1 from human lymphocytes. The four-parameter binding equation (Tijssen, 1985, Practice and Theory of Enzyme Immunoassays: Laboratory Techniques in Biochemistry and Molecular Biology 15:, New York: Elsevier Science Publishers)

$$A=(A_{max}-A_{min})/(1+(C/C_{1/2})b)+A_{min}$$

where A is the absorbance caused by a sample of concentration C, and $A_{max}$, $A_{min}$, $C_{1/2}$, and b are adjustable parameters, was fit to the standard curve by a nonlinear least squares algorithm, and was used to convert sample absorbances to DR1 concentrations.

For determination of the reactivity of DR1 from insect or human cells with a panel of anti-DR1 antibodies, a direct-

binding ELISA was used. Microtiter plates were coated with 200 ng per well of DR1 and blocked as above. Serial dilutions of monoclonal antibodies or biotinylated monoclonal antibodies were added to the plate; and bound antibodies were detected by alkaline-phosphatase goat anti-mouse antibodies, or with streptavidin-alkaline phosphatase, and p-nitrophenylphosphate (Harlow and Lane, 1988, Antibodies: A Laboratory Manual, Cold Spring Harbor, N.Y.). The antibody dilution that produced one-half the maximal absorbance was used to compare the affinity of each antibody for human- and insect-cell-derived DR1.

Isolation of DR1 from Coinletted Insect Cells

The procedures used to purify DR1 from insect cells were based on those developed for isolation of DR1 from human lymphoblastoid cells (Gorga et al., 1987, J. Biol. Chem. 262: 16087). Soluble DR1 was isolated from the conditioned culture medium of Sf9 insect cells coinfected with DRαsol and DRβsol. At 72 hr postinfection, cells were removed by centrifugation and the mixture of protease inhibitors was added. The culture medium was concentrated approximately 10-fold using a spiral membrane cartridge (Amicron SLY10) and used for immunoaffinity purification.

Soluble DR1 was also isolated from lysates of cells coinfected with DRαsol and DRβsol. Washed cells were lysed in 10 mM Tris-Cl (pH 8.0) containing the protease inhibitor mixture, by repeated passage through a 23 gauge needle. The lysate was centrifuged (200,000×g, 30 min, 4° C.) and the clarified lysate was enriched for DR1 by ion exchange chromatography on Q-Sepharose (Pharmacia) in 10 mM imidazole-HCl (pH 6.4) using a 50–250 mM NaCl gradient. DR1-containing fractions were pooled and used for immunoaffinity purification.

Full-length DR1 was isolated in detergent solution from BV-DRα+BV-DRβ-coinfected Sf9 insect cells. Washed cells were lysed with 1% CHAPS in PBS containing the protease inhibitor mixture. Nuclei and insoluble materials were removed by low-speed centrifugation (2,500×g, 5 min, 4° C.). The supernatant was cleared by ultracentrifugation (200,000×g, 30 min. 4° C.) and used for immunoaffinity purification.

Immunoaffinity matrices were prepared using anti-native DR1 monoclonal antibodies LB3.1 or L243. Purified antibodies were coupled at 5 mg/ml to protein A-agarose (Repligen) or to protein G-Sepharose Fast Flow using dimethyl pimelimidate as described (Harlow and Lane, 1988, Supra). Samples for immunoaffinity purification were passed through uncoupled protein A or protein G columns before application to the immunoaffinity column. Immunoaffinity columns were washed with PBS, and DR1 was eluted with 50 mM sodium cycloheylaminepropanesulfonate (CAPS) buffer (pH 11.5). Eluted fractions were immediately neutralized with 100 mM sodium phosphate (pH 6.0). Protein-containing fractions were pooled and concentrated into PBS using a spin ultrafiltration device (Centricon-30, Amicon). For purification of the full-length protein from BV-DRα+DV-DRβ-coinfected cells, all solutions contained 1% CHAPS. The concentration of purified DR1 was determined by ultraviolet absorbance at 280 nm using an extinction coefficient of 77,000 $M^{-1}cm^{-1}$.

N-terminal sequence analysis of purified soluble DR1 from insect cells was performed after separation of the subunits by SDS-PAGE and transfer to PVDF, by automated Edman degradation, as described (Matsudaira, 1987, J. Biol. Chem. 262: 10035).

Glycosylation Analysis

For glycosidase analysis, purified DR1 samples were denatured by boiling in 1% SDS plus 1% β-mercaptoethanol, then cooled and diluted 10-fold into PBS containing protease inhibitors and 1% dodecylmaltoside. Endoglycosidase-H (EC 3.2.1.96, 0.005 U per mg of DR1), or glycopeptidase-F (EC3.2.2.18, 1U per mg of DR1), or an equivalent volume of PBS, was added, and the mixtures were incubated at 37° C. for 12 hr. The reactions were stopped by again boiling in SDS and the reaction products were analyzed by 12.5% acrylamide SDS-PAGE. For lectin analysis, purified DR1 samples were analyzed as described above for Western blotting. Parallel blots were incubated with each of the digoxigenin-labeled lectins, and then with alkaline phosphatase-labeled anti-digoxigenin, and were developed as described above. DRα and DRβ bands were identified by comparison with parallel blots analyzed with rabbit anti-DRα and anti-DRβ sera.

Peptide Binding to Purified DR1

Immunoaffinity-purified, soluble DR1 (0.05–1.0 μM) from insect or human cells was used in binding reactions, with a 2- to 10-fold molar excess HA(306–318) peptide. Standard binding conditions were 37° C. for >72 hr in PBS (pH 7.2) with 1 mM EDTA, 1 mM PMSF, 0.1 mM iodoacetamide, and 3 mM $NaN_3$. Incubation time, pH and buffer were varied in some experiments (see figure legends). SDS-PAGE analyses were performed as described above, except that larger gels (14×14×0.15 cm) were used, and some samples were not boiled prior to loading, as noted in the figure legend. After electrophoresis, proteins were detected with Coomassie brilliant blue R-250. HPLC analyses were performed using a 7.8×300 mm Waters Protein-Pak SW300 gel filtration column, equipped with a Waters 1–125 guard column, and variable wavelength absorbance detector. PBS was used as the mobile phase, with a flow rate of 0.5 ml/min.

For quantitation of peptide binding, $[^{125}I]$HA(306–318) peptide was used. Peptide (10 μg) was radiolabeled with 1 mCi of $Na[^{125}I]$ and 50 μg of chloramine-T in phosphate buffer in a total volume of 50 μl for 2 min at room temperature, the reaction was stopped by the addition of excess $Na_2S_2O_5$, and the peptide was isolated by gel filtration over Sephadex G-15 (Pharmacia) in PBS. Peptide concentration in the labeled preparations was determined using a bicinchoninic acid assay by comparison with dilutions of an unlabeled peptide standard. Specific activities of the labeled peptide were 30,000–160,000 cpm/pmol in different preparations. Peptide bound to DR1 was separated from free peptide by HPLC gel filtration (as above), immunoabsorption, or spin ultrafiltration. Bound $^{125}I$-labeled peptide was measured by gamma counting.

For immunoabsorption, polystyrene microtiter wells (RIA/EIA 8-well strips, Costar) were coated overnight with 2 μg of purified L243 in 50 mM sodium carbonate (pH 9.6) and blocked with 5% nonfat dry milk. Milk was used to reduce nonspecific absorption, rather than BSA as in the ELISA assay, since radiolabeled HA(306–318) showed some binding to BSA. The DR1 binding capacity of these plates was determined to be 50 ng per well, and they were always used with subsaturating DR1 concentrations. Peptide binding mixtures (in triplicate) were added to an equal volume of blocking solution in the antibody-coated wells and were allowed to bind for 1 hr at room temperature. The wells were washed five times with PBST before gamma counting. For spin ultrafiltration, DR1 and DR1-peptide complexes were separated from free peptide by five cycles of concentration and 25-fold dilution into PBS, using Centricon-10 ultrafiltration devices (Amicon). Before use, the Centricon-10 devices were blocked with 5% nonfat dry milk and washed with PBS.

Isolation and Crystallization of Soluble DR1-Peptide Complexes

Immunoaffinity-purified soluble DR1 (0.5–1 mM) was incubated with 2- to 5-fold molar excess HA(306–318) peptide and with 1 mM EDTA, 1 mM PMSF, 0.1 mM iodoacetamide, and 3 mM $NaN_3$ at 37° C. for >72 hr. DR1-peptide complexes were separated from free peptide, aggregated DR1, and residual contaminating protein by gel filtration HPLC. The sharp peak corresponding to a molecular weight of about 50,000 was collected and concentrated by spin ultrafiltration. For crystallization, DR1 peptide complexes (5 mg/ml) were transferred to 10 mM Tris-Cl, 0.01% $NaN_3$ (pH 8.0). Crystals were obtained by vapor diffusion against 14–17% PEG 8000, 100 mM glycine (pH 3.5), using hanging drops on silanized microscope cover slips over precipitant solution in 24-well tissue culture plates.

Elution and Measurement of Bound Peptides

A procedure similar to that published for elution of peptides from class I MHC (Van Bleek and Nathanson, 1990, Nature 348: 213; Falk et al., 1991, Nature 351: 290; Jardetzky et al., 1991, Nature 353: 326) was used to elute DR1-associated peptides. DR1 samples (50 μg) were separated from low-molecular-weight material by gel filtration HPLC as above, except that 170 mM aqueous ammonium acetate was used as the mobile phase. DR1-containing fractions from each sample were pooled, and any residual low-molecular-weight material was removed by three cycles of 25-fold concentration and dilution into 170 mM aqueous ammonium acetate using a Centricon-30 ultrafiltration device as above. Bound peptides were eluted from the final concentrate by 25-fold dilution into 10% acetic acid and incubation at 70° C. for 15 min. The samples were cooled and concentrated once again. The final filtrate provided the pool of peptides eluted by acid denaturation. Filtrates were concentrated to 100 μl by vacuum centrifugation, and a portion was used for amino acid analysis on an ABI 420A/130A derivatizer/HPLC after hydrolysis with 6N HCl for 24 hr in vacuo. A sample of 170 mM ammonium acetate was processed in parallel through the HPLC, washing, elution, and analysis steps, to control for background and nonpeptidic reactivity.

Results

Recombinant Baculoviruses Direct the Synthesis and Secretion of DRαβ Heterodimers in Coinfected Sf9 Cells

Recombinant baculoviruses carrying full-length genes for the α and β subunits of human DR1 and (BV-DRα and DV-DRβ), or carrying truncated genes (BV-DRαsol and BV-DRβsol), were generated by homologous recombination in the insect ovarian cell line Sf9 (fall armyworm, *Spodoptera frugiperda*). The truncated genes code for proteins of 192(α) and 198(β) residues, which terminate just before the beginning of the predicted transmembrane spans (FIG. 1, bottom panels). Insect cells infected with BV-DRα or with BV-DRβ expressed polypeptides of the expected apparent molecular weight, which reacted with antisera specific for the appropriate subunit of DR1 (FIG. 1, lanes 5). No reactivity was observed in the extracellular medium (FIG. 1, lanes 6), nor in insect cells infected with a control baculovirus, BV-μgal (FIG. 1, lanes 3 and 4).

Insect cells infected with BV-DRαsol or BV-DRβsol, which carry the truncated genes, expressed polypeptides that exhibited somewhat faster mobility on SDS-PAGE (FIG. 1, lanes 7) than the full-length forms, as expected for the removal of the transmembrane and cytoplasmic domains. The truncated constructs were expressed at a significantly greater level than the full-length proteins. A fraction of the protein produced in the singly-infected cells was secreted into the extracellular medium (FIG. 1, lanes 8). The protein retained within the cells exhibited multiple bands per subunit by SDS-PAGE, probably due to incomplete signal sequence cleavage and partial glycosylation, but the secreted protein exhibited predominantly one band per subunit. In cells coinfected with both BV-DRαsol and DV-DRβsol, secretion into the extracellular medium was much more efficient (FIG. 1, lanes 9 and 10).

The differences in mobility on SDS-PAGE between the subunits of DR1 expressed in insect cells and those of full-length DR1 (FIG. 1, lanes 1) or papain-solubilized DR1 (FIG. 1, lanes 2) produced by human cells are due to differential glycosylation in the insect cell and human cell lines. Both the α and β chains of the full-length and truncated forms of DR1 from insect cells were sensitive to endoglycosidase-H and glycopeptidase-F (Table 1). Both α and β chains bound GNA lectin but not SNA, MAA, DSA, or PNA lectins, indicating that both chains contain high-mannose, N-linked polysaccharides. In contrast, DR1 isolated from human cells carries a complex, sialated polysaccharide on each chain, along with a second, nonsialated polysaccharide on the α chain (Table 1 and Shackelford and Strominger, 1983, J. Immunol. 130: 274). After deglycosylation, the subunits of full-length and truncated DR1 from insect cells exhibited the same mobility as the corresponding deglycosylated subunits of intact or papain-solubilized DR1 from human cells.

Sf9 insect cells coinfected with the full-length constructs, BV-DRα+BV-DRβ, express DR1 on the cell surface as detected by flow cytometry using monoclonal antibody L243 (FIG. 2C). This antibody recognizes a conformational determinant on the correctly folded DR1 heterodimer (Lampson and Levy, 1980, J. Immunol. 125: 293; Gorga et al., 1987, J. Biol. Chem. 262: 16087). No reactivity was observed with Sf9 cells singly infected with BV-DRα alone (FIG. 2A) or with BV-DRβ alone (FIG. 2B). The surface expression of DR1 on the coinfected SF9 insect cell surface was weaker and more heterogeneous than that exhibited by LG2, a human lymphoblastoid cell line (FIG. 2D).

The time course of DR1 expression in insect cells was monitored by enzyme-linked immunosorbent assay (ELISA) using the anti-native DR1 monoclonal antibody L243 (FIG. 3). DR1 expression in BV-DRα+BV-DRβ-coinfected cells increased from 24 to 48 hr postinfection, then remained relatively constant (closed squares). DR1 could not be detected in the extracellular medium (open squares). No L243 reactivity was observed in lysates of singly-infected cells (shaded triangles), indicating that this antibody does not recognize DRα or DRβ monomers, or any $\alpha_2$ or $\beta_2$ homodimers that may be produced by the singly infected cells. Similar results were obtained with LB3.1 (Gorga et al., 1986, Cell. Immunol. 103: 160), another conformation-sensitive monoclonal antibody that recognizes the DRαβ heterodimer. Insect cells coinfected with the truncated constructs, BV-DRαsol and BV-DRβsol, produced heterodimeric DRαβ complex that was detected in cell lysates (closed circles) and also in the extracellular medium (open circles). Secretion of DR1 to the extracellular medium significantly lagged behind expression within the cell and continued to increase very late in infection. The overall expression level of soluble DR1 (cells plus medium) remained fairly constant after 48 hr postinfection, at approximately 2 mg per liter of culture medium, more than six times the expression level of the full-length, membrane-bound form.

Purification of DR1 from Insect Cells

Insect cell cultures were harvested for protein purification at 72 hr postinfection. Soluble DR1 (1–2 μg per ml of culture) was isolated from the extracellular medium of

coinfected cells in 80%–90% yield by immunoaffinity chromatography using monoclonal antibodies that recognize the native DR1 heterodimer (LB3.1 or L243). No DRα or DRβ subunits could be detected on Western blots of the material that did not bind to the affinity column, indicating that all of the secreted DRα and DRβ was present as αβ heterodimer. The immunoaffinity-purified soluble DR1 exhibited predominantly two bands (DRα and DRβ) by SDS-PAGE, along with a significant but variable amount of a second DRα band (FIG. 4, lane 1). The three bands were subjected to N-terminal sequencing. Both DRα bands had the sequence $NH_2$-IKEEH . . . , and the DRβ band had the sequence $NH_2$-GDTRP . . . These are the N-termini expected for the mature subunits, indicating that the native DR1 signal sequences were efficiently removed by the insect cell. The purified DR1 was tested against 13 monoclonal antibodies that recognize native DR1 from human cells. Each of the antibodies tested, DA2(β1-specific), DA6.147(α), DA6.231(β1), IVA12(β1), L227(β1), SG171(β1), TAL8.1(β1), TAL14.1(β1), Tu36(β2), Tu39(β1), and Tu43(αβ), as well as the antibodies used for affinity purification L243(α) and LB3.1(α), bound to the soluble, insect-cell-derived DR1.

Approximately half of the total soluble DR1 produced by BV-DRαsol+BV-DRβsol-coinfected cells was retained within the cells. This material could be isolated from a lysate of coinfected cells by ion exchange and immunoaffinity chromatographies. Soluble DR1 isolated from cell lysates behaved similarly to soluble DR1 isolated from the extracellular medium. Full-length DR1 (0.1 μg/ml of culture) could be isolated in detergent solution from lysates of BV-DRα+BV-DRβ-coinfected cells, by including 1% 3-[(3-choamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) in all solutions throughout the purification procedure.

Purified Soluble DR1 is Stabilized by Antigenic Peptide

DR1 isolated from human lymphoblastoid cells is substantially resistant to dissociation by SDS at room temperature, and the α and β chains migrate as a heterodimer on SDS-PAGE if the samples are not boiled prior to loading (FIG. 4, lane 8; Gorga et al., 1987, J. Biol. Chem. 262: 16087). After boiling in SDS, the α and β subunits dissassociate (FIG. 4, lane 7). In contrast, the soluble DR1 secreted from coinfected insect cells was sensitive to dissociation by SDS at room temperature, and migrated mostly as monomeric α and β chains, along with several faint bands near the position expected for the heterodimer (FIG. 4, lane 4). After boiling, soluble DR1 from insect cells migrated as the expected α and β monomers (FIG. 4, lane 3). Preincubation with an antigenic peptide from influenza hemagglutinin, HA(306–318), caused the soluble DR1 from insect cells to become resistant to SDS-induced dissociation. Soluble DR1 from insect cells, incubated with HA(306–318) peptide and subsequently treated with SDS at room temperature (FIG. 4, lane 2), migrates as a strong band that corresponds to the αβ heterodimer seen with DR1 from human cells. After boiling, the subunits dissociate (FIG. 4, lane 1). Incubation of DR1 from human lymphoblastoid cells with peptide had no effect on the stability to SDS-induced dissociation (FIG. 4, lanes 5 and 6).

While the DR1 isolated by immunoaffinity purification appeared to be substantially free of contaminating proteins by SDS-PAGE, it eluted from a gel permeation column in a number of peaks with apparent molecular weights of 50,000 and greater (FIG. 5A). Each of these peaks contained material that reacted with anti-DRαβ antibodies. After incubation for 72 hr at 37° C. with antigenic peptide HA(306–318), most of the protein eluted in a single peak corresponding to 50,000 daltons (FIG. 5B), as expected for the DRαβ heterodimer and as seen with DR1 isolated from human lymphoblastoid cells (FIG. 5C). Incubation of DR1 from insect cells without the addition of peptide had no effect on its aggregation behavior. The aggregation was not a result of the isolation procedure, as whole conditioned medium also exhibited multiple, DR1-containing peaks. [$^{125}$I]HA(306–318) peptide included with soluble DR1 from insect cells in the incubation mixture comigrated with the strong DR1 αβ peak (FIG. 5D, open bars). Radiolabeled peptide binding could be competed with an excess of unlabeled peptide (solid bars). The effect of added peptide in converting the heterogenous DR1 isolated from insect cells (FIG. 5A) to a mostly homogeneous species (FIG. 5B) thus occurs through peptide binding to the DR1 molecule.

Gel filtration HPLC was used to isolate the complex of soluble, insect-cell-derived DR1 with HA(306–318) peptide. The purified DR1-peptide complex retained binding to all of the anti-DR1 monoclonal antibodies described above. DR1-HA(306–318) peptide complexes were crystallized by vapor diffusion from polyethylene glycol, under conditions previously developed for crystallization of DR1 from human cells (Gorga et al., 1991, Res. Immunol. 142: 401) These crystals were morphologically similar to those produced from papain-solubilized DR1 isolated from human lymphoblastoid cells.

The SDS-PAGE (FIG. 4) and HPLC gel filtration (FIG. 5) results indicate that DR1 isolated from insect cells was less stable to denaturation and aggregation that DR1 isolated from human lymphoblastoid cells. In both assays, preincubation of the insect-cell-derived DR1 with antigenic peptide caused it to behave similarly to DR1 from human cells. In contrast, incubation of human-cell-derived DR1 with peptide had no effect on its behavior in HPLC gel filtration or SDS-PAGE, presumably because the protein as isolated is already saturated with tightly bound peptides.

Binding of Antigenic Peptide to Soluble DR1 from Insect Cells

The kinetics of radioiodinated HA(306–318) peptide binding to DR1 were measured at pH 7.2 and 37° C. for soluble DR1 produced by coinfected insect cells (FIG. 6, left panel, squares) and by human lymphoblastoid cells (circles). The initial rate of peptide binding to insect-cell-derived DR1 was 0.11 mol peptide per mole DR1 per hour, significantly faster than the 0.0093 mol peptide per mole DR1 per hour observed for human-cell-derived DR1. These initial rates correspond to pseudo-first-order rate constants of 12 $M^{-1}s^{-1}$ for DR1 from insect cells and 1.0 $M^{-1}s^{-1}$ for DR1 from human cells. The extent of radiolabeled HA(306–318) peptide binding to DR1 from insect and human cells was determined from the data in FIG. 6 (left panel). At times after 24 hr, the amount of peptide bound to the insect-cell-produced DR1 was 1.0±0.3 mol peptide per mole DR1. For human-cell-produced DR1 the (extrapolated) maximum extent of binding was 0.2±0.1 mole peptide per mole DR1.

For measurement of dissociation kinetics (FIG. 6, right panel), DR1 samples were equilibrated with excess [$^{125}$I] HA(306–318) peptide for 73 hr at 37° C. After this time, DR1-peptide complexes were separated from free peptide, diluted into buffer containing excess unlabeled peptide, and returned to 37° C. Samples were removed at the indicated times, and the amount of peptide remaining bound to DR1 was measured. The kinetics of peptide dissociation were extremely slow for DR1 from either source, and no significant difference in dissociation rate were observed over 300 hr. The dissociation data for DR1 from both sources are

consistent with a first-order dissociation constant of about $4\times10^{-6}\ s^{-1}$.

The pH dependence of peptide binding of DR1 from human and insect cells was also determined. Binding of excess [$^{125}$I]HA(306–318) peptide to DR1 was measured after 72 hour incubation of 37° C., for soluble DR1 from human cells (FIG. 7, solid bars) and from insect cells (shaded bars). Open and hatched bars show binding in the presence of excess unlabeled peptide. Peptide binding to human lymphoblastoid-cell-derived DR1 increased at lower pH. In contrast, peptide binding to insect-cell-derived DR1 was relatively independent of pH. At every pH tested, insect cell DR1 bound more peptide than DR1 from human lymphoblastoid cells. The extent of peptide binding observed for the insect-cell-derived DR1 corresponds to 1.1±0.2 mol peptide per mole protein. For the DR1 isolated from human cells, the extent varied from 0.06 (pH 8) to 0.3 (pH 4).

The measurements of peptide binding capacity were repeated using different preparations of [$^{125}$I]HA(306–318) peptide and DR1 from insect and human cells (Table 2). Soluble DR1 produced in insect cells reproducibly bound nearly a stoichiometric amount of peptide (0.90±0.15 mol peptide per mole DR1), while DR1 from human cells bound 5-fold less peptide (0.17±0.07 mol peptide per mole DR1). The low binding capacity, slow association kinetics and pH dependence of peptide binding for class II molecules isolated from mammalian cells are all believed to be due to the presence of tightly bound peptides occupying the antigen-binding site, which must dissociate before exogenously added peptide will bind (Buus et al., 1986, Cell 47: 1071; Roche and Cresswell, 1990, J. Immunol. 144: 1799; Tampe and McConnell, 1991, Proc. Natl. Acad. Sci. USA 88: 4661). Taken together, the increased peptide binding capacity, increased binding rate, and decreased pH dependence of peptide binding for DR1 produced in insect cells indicate that, as isolated, the antigen-binding site is largely empty.

To confirm this result, we directly measured the amount of endogenous peptide bound to DR1, using a procedure previously used to characterize peptides bound to class I and class II molecules (Van Bleek and Nathanson, 1990, Nature 348: 213; Falk et al., 1991, Nature 351: 290; Rudensky et al., 1991, Nature 353: 662). A pool of bound peptides was released from the DR1-binding site by acid denaturation, isolated by spin ultrafiltration, and finally quantitated by amino acid analysis. Papain-solubilized DR1 isolated from human cells carried the equivalent of 14 amino acid residues per mole (Table 2). Full-length DR1 from human cells gave essentially the same result. This corresponds to approximately 95% occupancy, with endogenous peptides having an average length of 15 residues. As a control, soluble DR1 from insect cells was analyzed after loading with HA(306–318) peptide. The isolated DR1-peptide complexes carried 13 amino acid residues per mole DR1, consistent with the length of the HA(306–318) peptide and a 1:1 molar ratio of bound peptide to DR1. In contrast, no amino acid residues were detected in the pool from soluble DR1 from insect cells above the reactivity observed in a buffer blank.

Using the methods described above, an ordinary artisan skilled in the art can generate empty MHC class II heterodimers from any mammalian species that encodes such proteins, for example, a mouse a rat, or a rabbit etc. Heterodimers comprising an α and a β chain are known in these species. The artisan, following the directions described above for the expression of the human heterodimer, can clone the genes encoding α and β peptides from other species of mammals into a suitable plasmid and generate baculoviruses that encode one or other of the peptides. Insect



cells when coinfected with these baculoviruses should express either membrane-associated or soluble heterodimers which are empty, and which can be loaded with a suitable antigenic peptide according to the methods of the invention.

TABLE 1

Glycosylation of DR1 Produced in Human and Insect Cells

| Assay | Specificity | DR1 Reactivity Insect α | Insect β | Human α | Human β |
|---|---|---|---|---|---|
| Glycosidase sensitivity | | | | | |
| Endoglycosidase H | High Mannose or hybrid | + | + | +/− | − |
| Glycopeptidase F | Most N-linked | + | + | + | + |
| Lectin reactivity | | | | | |
| GNA (*Galanthus nivalis* agglutinin) | ManαMan | + | + | − | − |
| SNA (*Sambucus nigra* agglutinin) | SAα(2–6)Gal | − | − | + | + |
| MAA (*Maackia amurensis* agglutinin) | SAα(2–3)Gal | − | − | − | − |
| DSA (*Datura straminium* agglutinin) | Galβ(1–4)-GlcNAc | − | − | + | − |
| PNA (peanut agglutinin) | Galβ(1–3)-GalNAc | − | − | − | − |

For glycosidase analysis, purified DR1 samples were denatured, digested with the appropriate glycosidase, and analyzed by SDS-PAGE. A difference in mobility in the glycosidase-treated samples relative to mock-digested samples was scored as positive. For lectin analysis, purified DR1 samples were analyzed by SDS-PAGE and Western blotting using labeled lectins. In both assays, full-length and soluble DR1 behaved identically. The expected oligosaccharide specificity is shown beside the nam e of each glycosidase or lectin (Man, mannose: SA, sialic acid: Gal, galactose: GlcNAc, N-acetylglucosamine: GalNAc, N-acetylgalactosamine).

TABLE 2

Stoichiometry of Peptide Binding to HLA-DR1 from Human and insect Cells

| DR1 Source | [$^{125}$I]HA(306-318) Peptide Bound (mol peptide/mol DR1) | Peptide Released by Acid Treatment (mol amino acid residue/mol DR1) |
|---|---|---|
| Human | 0.17 ± 0.07 | 14 |
| Insect | 0.90 ± 0.1 | ND |
| Insect (pre-loaded | — | 13 |

The extent of [$^{125}$I]HA(306-318) peptide binding to soluble DR1 produced in insect cells and in human cells was determined for the experiments shown in FIGS. 5, 6, and 7 (pH 7 values only) and in four other trials. In each experiment, DR1 samples from insect and human cells were treated in parallel. Occupancy values for $^{125}$I-labeled HA peptide are given as the ratio of moles peptide bound per mole DR1, determined using the measured specific activity of the [$^{125}$I]HA(306-318) preparation and the concentration of DR1 determined by ELISA or absorbance at 280 nm. Values are the average of seven trials with the observed standard deviation. The amount of endogenous peptide bound to soluble DR1 was determined for papain-solubilized human DR1 and secreted insect cell DR1, and also for insect cell DR1 preloaded with HA(306-318) peptide. Bound peptides were released by acid treatment and isolated by spin ultracentrifugation. Occupancy values are given as the ratio of moles amino acid residue in the peptide fraction, determined by amino acid analysis, per mole DR1, determined by absorbance at 280 nm. ND, none detected above the background reactivity observed for a buffer blank. Detection limit was approximately 5 amino acid residues per mole DR1.

Advantages of the Invention

The biological and immunological properties of histocompatibility proteins are largely defined by the antigenic peptide that is bound to them. All previous methods for producing class II histocompatibility proteins have provided material that contains a mixture of antigenic peptides (Buus et al., 1988, Science, 242: 1045; Rudensky et al., 1991, Nature, 353, 662), which can be only partially loaded with a defined antigenic peptide (Watts and McConnell, 1986, Proc. Natl. Acad. Sci. USA 83: 9660; Buus et al., 1987, Immunol. Rev. 98: 115; Ceppellini et al., 1989, Nature 339: 392; Busch et al., 1990, J. Immunol. Meth. 134: 1; Jardetzky et al., 1990, EMBO J. 9: 1797; O'Sullivan et al., 1990, J. Immunol. 145: 1799; Roche and Cresswell, 1990, J. Immunol. 144: 1849). Previous efforts to produce soluble MHC class II heterodimers by recombinant methods have been unsuccessful (Traunecker et al., 1989, Immunol. Today, 10: 29). The methods described in the invention provide empty class II histocompatibility proteins, which can be completely loaded with any desired antigenic peptide. In addition, the methods provide soluble histocompatibility proteins without the use of proteases and provide better yields of histocompatibility protein than current methods. Furthermore, the process is more economical and allows the protein sequence to be manipulated in any desired manner.

Uses of the Invention

The compositions and methods of the invention are useful for the treatment of humans with any disease in which an immune response to a protein causes unwanted symptoms. Thus the compositions and methods of the invention may be used to treat autoimmune disease. During autoimmune disease, T cells activated by MHC class II self-antigenic peptide complexes initiate an immune response directed against the body's own antigenic peptides in tissues and organs. However, binding of T cells to large quantities of MHC-antigenic peptide complexes has been shown to have the opposite effect in that the T cells become inactivated (Quill and Schwartz, 1987, J. Immunol. 138: 3704). While the mechanism by which this inactivation is induced is not understood, two separate theories might explain this phenomenon. High concentrations of MHC-antigenic peptide complexes when bound to T cells may simply saturate T cell receptors for that complex, thus competitively blocking the T cells' ability to bind to an identical MHC-antigenic peptide complex present in tissues and organs. Similarly, although somewhat distinct, saturation of T cell receptors with MHC-antigenic peptide complexes may induce a state of clonal energy, wherein the clone of T cells to which the peptides are bound become incapable of activating subsequent immune events required for an immune response (Quill and Schwartz, 1987, J. Immunol. 138: 3704).

Using the methods and compositions described above it is now possible to prepare large quantities of membrane-associated or soluble MHC heterodimers that have bound to them an antigenic peptide of choice. Such an antigenic peptide might be one which triggers an autoimmune reaction in a patient with an autoimmune disease. Treatment of the patient with such MHC-antigenic peptide complexes may induce clonal anergy, or otherwise diminish or eliminate the T cell's ability to promote the autoimmune reaction.

The compositions and methods of the invention may also be used to specifically destroy autoreactive T cells. Heterodimer-antigenic peptide complexes, that are themselves conjugated to a toxin molecule, may be used to target the toxin to the autoreactive T cells, whereupon the toxin would then induce death of the T cells.

The heterodimer-antigenic peptide complex of the invention may also be used to vaccinate a patient with an antigenic peptide that when administered to the patient in the absence of the heterodimer, is incorrectly processed by the antigen-presenting cells in the body. The heterodimerantigenic peptide complex may be administered to the patient, either in solution or attached to a solid support, as an artificial antigen-presenting cell, capable of inducing a protective immune response in the patient.

The compositions and methods of the invention may also be used as a research or a diagnostic tool to identify the presence of, and to isolate T cells that are reactive with a particular heterodimer-antigen complex. In order to determine the origin and function of clonal lines of T cells and to examine their role in autoimmune disease, it is often necessary to isolate these cells in a pure form, i.e., separated from all other cells in the population, including other T cells of a different clonal origin. The compositions and methods of the invention can easily be used to isolate T cells that are reactive to a specific self-antigen, without having to go through the more conventional yet cumbersome process of first obtaining a monoclonal antibody directed against the particular T cell receptor epitope expressed by those T cells. Briefly, a population of lymphocytes are obtained from a mammal by conventional means. The self-antigen in question is complexed to empty heterodimers using the methods described above. The heterodimer has a dye, e.g., a fluorescent dye, conjugated to it using methods standard to those in the art. For example, conjugation can be accomplished using standard methods for conjugation of dyes to antibodies such as those described in Stites and Terr (1991, Basic and Clinical Immunology, Appleton and Lange) or in Harlow and Lund (Supra). The lymphocytes are incubated in the presence of heterodimer-antigen complex and only T cells that are reactive to the self-antigen present in the heterodimer complex will bind to the complex, thus becoming labeled with the dye. Labeled cells are then separated from unlabeled cells by conventional cell-sorting flow cytometry. Thus the compositions and methods of the invention provide a rapid and easy method for the purification of T cell clones that are reactive to self-antigens.

In a manner similar to that described above, the compositions and methods of the invention can be used as a diagnostic tool determine the onset of autoimmune disease in a patient, and/or to follow the progress of the disease in that patient. For example, lymphocytes obtained from a patient can be reacted with dye-labeled heterodimer-self-antigen complex without further purification. Cells that have either bound the complex or not bound the complex can be separated from free unbound complex by several cycles of centrifugation and washing. The cells can then be examined by fluorescence microscopy for the presence of the dye. If the cells are counterstained with a dye of a different color which stains all cells, for example rhodamine or Texas red, then it is possible to quantitate the number of T cells which have bound the heterodimer. Similarly, quantitation can be accomplished using flow cytometry as described above. Thus the presence of and the number of self-antigen reactive T cells can be determined in a sample obtained from a patient suspected of having an autoimmune disease. In order to monitor progression of the disease in a patient, or to monitor T cell activity in patients receiving treatment for the disease, samples can be obtained periodically and analyzed as described above. Such sampling, which in the majority of cases will involve obtaining circulating lymphocytes from the patient's blood, is a relatively painless and non-invasive procedure.

Use of the compositions and methods of the invention is not limited to the study of autoimmune disease in humans.

Where animal models of autoimmune disease are available, or become available, the compositions and methods of the invention provide an invaluable research tool to further examine the disease process, thus generating information that can then be used to eliminate or diminish the severity of such a disease in humans.

Diseases that are potentially treatable using the compositions and methods of the invention, all of which have been linked to the major histocompatibility class II molecules, are presented below. The autoreactive antigenic peptide, where it is known or suspected, is given in parentheses. The actual antigenic peptide used for forming the complex with class II might be derived from these peptides.

Multiple sclerosis (myelin basic protein)
Myasthenia gravis (acetylcholine receptor)
Systemic lupus erythematosus (DNA)
Glomerulonephritis or Goodpasture's syndrome (type IV collagen)
Insulin-dependent diabetes mellitus (insulin receptor)
Autoimmune hemolytic anemia (erythrocyte membrane proteins)
Autoimmune thrombocytopenic purpura (platelet membrane proteins)
Grave's disease (thyroid stimulating hormone receptor)
Pernicious anemia
Rheumatoid arthritis
Dermatitis herpetiformis
Celiac disease
Sicca syndrome
Idiopathic Addison's disease
Idiopathic membranous nephropathy
Narcolepsy
Optic neuritis
Postpartum thyroiditis
Hashimoto's thyroiditis
Juvenile rheumatoid arthritis.

MHC heterodimer-antigenic peptide complexes can be administered parenterally, for example intravenous, subcutaneous, intramuscular, intraorbital or intraocular administration. The complexes can be formulated for parenteral administration to humans or other mammals in therapeutically effective amounts (e.g., amounts which eliminate or reduce the patient's pathological condition) to provide therapy for the diseases described above.

The complexes provided herein can be formulated into pharmaceutical compositions by admixture with pharmaceutically acceptable non-toxic excipients and carriers. Such compositions may be prepared for use in parenteral administration, particularly in the form of liquid solutions or suspensions.

The complexes may conveniently be administered in unit dosage form and may be prepared by any of the methods well known in the pharmaceutical art, for example as described in *Remington's Pharmaceutical Sciences*. Formulations for administration may contain as common excipients sterile water or saline, cyclodextrans, polyalkylene glycols, such as polyethylene glycol, oils of vegetable origin, hydrogenated naphthalenes and the like. In particular, biocompatible, biodegradable lactide polymer, lactide/glycolide copolymer, or polyoxyethylene-polyoxypropylene copolymers may be useful excipients to control the release of the peptides. Other potentially useful delivery systems for these complexes include ethylene-vinyl acetate copolymer particles, osmotic pumps, implantable infusion systems, and liposomes. Formulations for administration may include a stabilizing agent, such as human serum albumin, as well as a permeation enhancer, such as glycocholate.

The concentrations of the complexes described herein in a therapeutic composition will vary depending upon a number of factors, including the dosage of the complex to be administered and can be determined on a case by case basis.

SEQUENCE LISTING

( 1 ) GENERAL INFORMATION:

( i i i ) NUMBER OF SEQUENCES: 9

( 2 ) INFORMATION FOR SEQ ID NO:1:

( i ) SEQUENCE CHARACTERISTICS:
( A ) LENGTH: 102 base pairs
( B ) TYPE: nucleic acid
( C ) STRANDEDNESS: single
( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: DNA (genomic)

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GGGTGGAGCA CTGGGGCTTG GATGAGCCTC TTCTCAAGCA TTGGGAATTC GATGCTCCAA 60

GCCCTCTCCC AGAGACTACA GAGAACTAAG CGGCCGCGGT AC 102

( 2 ) INFORMATION FOR SEQ ID NO:2:

( i ) SEQUENCE CHARACTERISTICS:
( A ) LENGTH: 102 base pairs
( B ) TYPE: nucleic acid
( C ) STRANDEDNESS: single
( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: DNA (genomic)

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CGCGGCCGCT TAGTTCTCTG TAGTCTCTGG GAGAGGGCTT GGAGCATCGA ATTCCCAATG 60

CTTGAGAAGA GGCTCATCCA AGCCCCAGTG CTCCACCCTG CA 102

( 2 ) INFORMATION FOR SEQ ID NO:3:

( i ) SEQUENCE CHARACTERISTICS:
( A ) LENGTH: 39 base pairs
( B ) TYPE: nucleic acid
( C ) STRANDEDNESS: single
( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: DNA (genomic)

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GACTTGGATC CTATAAATAT GGTGTGTCTG AAGCTCCCT 39

( 2 ) INFORMATION FOR SEQ ID NO:4:

( i ) SEQUENCE CHARACTERISTICS:
( A ) LENGTH: 35 base pairs
( B ) TYPE: nucleic acid
( C ) STRANDEDNESS: single
( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: DNA (genomic)

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:4:

ACAGCTCTAG ATTACTTGCT CTGTGCAGAT TCAGA 35

( 2 ) INFORMATION FOR SEQ ID NO:5:

( i ) SEQUENCE CHARACTERISTICS:
( A ) LENGTH: 13 amino acids
( B ) TYPE: amino acid
( C ) STRANDEDNESS: Not Relevant
( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: protein

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Pro Lys Tyr Val Lys Gln Asn Thr Leu Lys Leu Ala Thr
1 5 10

( 2 ) INFORMATION FOR SEQ ID NO:6:

( i ) SEQUENCE CHARACTERISTICS:
( A ) LENGTH: 11 amino acids
( B ) TYPE: amino acid
( C ) STRANDEDNESS: Not Relevant
( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: protein

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Pro Glu Thr Thr Glu Asn Val Val Cys Ala Leu
1 5 10

( 2 ) INFORMATION FOR SEQ ID NO:7:

( i ) SEQUENCE CHARACTERISTICS:
( A ) LENGTH: 6 amino acids
( B ) TYPE: amino acid
( C ) STRANDEDNESS: Not Relevant
( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: protein

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:7:

-continued

Pro Glu Thr Thr Glu Asn
1 5

( 2 ) INFORMATION FOR SEQ ID NO:8:

( i ) SEQUENCE CHARACTERISTICS:
( A ) LENGTH: 11 amino acids
( B ) TYPE: amino acid
( C ) STRANDEDNESS: Not Relevant
( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: protein

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Glu Ser Ala Gln Ser Lys Met Leu Ser Gly Val
1 5 10

( 2 ) INFORMATION FOR SEQ ID NO:9:

( i ) SEQUENCE CHARACTERISTICS:
( A ) LENGTH: 6 amino acids
( B ) TYPE: amino acid
( C ) STRANDEDNESS: Not Relevant
( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: protein

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Glu Ser Ala Gln Ser Lys
1 5

I claim:

**1**. An isolated sample of mammalian major histocompatibility class II heterodimers capable of binding 1.0+/−0.3 mol antigenic peptide per mol heterodimer when said antigenic peptide is added to said sample, wherein said sample is produced by expressing DNA encoding the α and β polypeptides of said major histocompatibility class II heterodimer in an insect cell.

**2**. The sample of claim **1**, wherein said heterodimers are soluble and the α and β polypeptides of each of said heterodimers lack the transmembrane domain normally present on naturally occurring major histocompatibility class II α and β polypeptides.

**3**. The sample of claim **1** or **2**, wherein said heterodimers are human.

**4**. The sample of claim **1** or **2**, wherein said heterodimers are murine.

**5**. A baculovirus comprising DNA encoding the α polypeptide of a mammalian major histocompatibility class II heterodimer.

**6**. The baculovirus of claim **5**, wherein said α polypeptide lacks the transmembrane domain normally present on naturally occurring α polypeptide.

**7**. The baculovirus of claim **5**, wherein said baculovirus is BV-DRα.

**8**. The baculovirus of claim **6**, wherein said baculovirus is BV-DRαsol.

**9**. A baculovirus comprising DNA encoding the β polypeptide of a mammalian major histocompatibility class II heterodimer.

**10**. The baculovirus of claim **9**, wherein said β polypeptide lacks the transmembrane domain normally present on naturally occurring β polypeptide.

**11**. The baculovirus of claim **9**, wherein said baculovirus is BV-DRβ.

**12**. The baculovirus of claim **10**, wherein said baculovirus is BV-DRβsol.

**13**. A method of producing the sample of claim **1**, said method comprising

expressing the α and β polypeptides of the mammalian major histocompatibility class II heterodimer in insect cells which comprise a baculovirus encoding the alpha polypeptide of a mammalian major histocompatibility class II heterodimer and a baculovirus encoding the beta polypeptide of a mammalian major histocompatibility class II heterodimer, and

recovering said heterodimer from said cells or their growth medium.

**14**. The method of claim **13**, wherein said heterodimer is soluble and said cells are coinfected with a baculovirus encoding the alpha polypeptide of a mammalian major histocompatibility class II heterodimer, wherein said alpha polypeptide lacks the transmembrane domain normally present on naturally occurring alpha polypeptide, and a baculovirus encoding the beta polypeptide of a mammalian major histocompatibility class II heterodimer, wherein said beta polypeptide lacks the transmembrane domain normally present on naturally occurring beta polypeptide.

**15**. A recombinant insert cell which expresses a mammalian major histocompatibility class II heterodimer which lacks bound antigen.

**16**. The cell of claim **15**, wherein said heterodimer is soluble and each of the α and β polypeptides of said heterodimer lacks the transmembrane domain normally present on naturally occurring major histocompatibility class II α and β polypeptides.

**17**. The cell of claim **15** wherein said cell is coinfected with the a baculovirus encoding the alpha polypeptide of a mammalian major histocompatibility class II heterodimer and a baculovirus encoding the beta polypeptide of a mammalian major histocompatibility class II heterodimer.

**18**. The cell of claim **16**, wherein said cell is coinfected with the a baculovirus encoding the alpha polypeptide of a

mammalian major histocompatibility class II heterodimer, wherein said alpha polypeptide lacks the transmembrane domain normally present on naturally occurring alpha polypeptide, and a baculovirus encoding the beta polypeptide of a mammalian major histocompatibility class II heterodimer, wherein said beta polypeptide lacks the transmembrane domain normally present on naturally occurring beta polypeptide.

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